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Do Mosquito Pesticides Harm Their Natural Enemies? Ecological Impacts and Non-Target Effects of Larvicides on Mosquito Predators

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DO MOSQUITO PESTICIDES HARM THEIR NATURAL ENEMIES? ECOLOGICAL IMPACTS AND NON-TARGET EFFECTS OF LARVICIDES ON MOSQUITO PREDATORS

by

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ABSTRACT

Larvicides are chemicals used to kill juvenile mosquitoes. When applied to an area, other aquatic organisms are exposed to these chemicals. The removal or impairment of top insect predators could be beneficial to mosquito populations once harmful pesticide levels dissipate. Two common larvicides were examined: growth regulators (IGRs) and surface films (SFs). The goal of this project was to determine if larvicides harm mosquito predators common to southern Mississippi.

I surveyed aquatic sites before and after IGR and SF treatments, and then compared changes in insect community structure. Community evenness was lower in SF treated habitats. When analyzing prey taxa only, evenness and diversity changed in control treatments, which suggests that differences measured were due to other environmental factors, not larvicide presence.

I examined lethal and behavioral effects of IGRs and SFs on predatory insects. Surface films were lethal to *Laccophilus* adults (Coleoptera: Dytiscidae) at recommended and high concentrations. Dragonfly nymph location preference in aquariums varied between SFs and IGRs. *Laccophilus* larvae in IGRs spent more time moving and eating compared to SFs. Behavioral differences were among combined concentrations in both larvicide treatments, not within their respective concentrations and controls.

Experiments were conducted to determine IGR and SF effects on the mosquito-regulating ability of predaceous insects. Treated predators were placed in mesocosms containing mosquito larvae. Mosquito survival was quantified by capturing emerging adults. There were no differences in emergence among all treatments. Implications of the

findings from this thesis, similarities to past research, and suggestions for future work are discussed.

ACKNOWLEDGEMENTS

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I would also like to thank Catherine Dean for her expertise in mosquito identification, advice, and help with countless laboratory and field tasks. Thank you to Nicole Scavo for her assistance and tips in my analyses in R. Also, thanks to Rachel Rogers for helping me with the test-runs of my behavioral experiments. Thank you to the Sigma-Xi Honor Society for the research support grant, which allowed me to travel to my field sites throughout the summer and fall of 2018. Lastly, thank you to all my colleagues at USM for their support.

DEDICATION

To my fiancé Cara and my family, Marc, Sandy, Kate. I would not be where I am today without their support throughout my time as an undergraduate and master's student.

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1.1 Introduction

The control of mosquito species that vector pathogens and the minimization of disease outbreaks is becoming increasingly important as human populations grow and urbanization expands. Effective ways to reduce adult mosquitoes include removing their aquatic rearing sites in proximity to people or targeting or removing their larvae in these standing water habitats. Understanding more about anthropophilic mosquito biology, their ecological importance, and the effectiveness of new abatement measures will help with future control of mosquito vectored disease. Currently, integrated mosquito control practices use a combination of surveying, source reduction, public education, biological control, and pesticides like adulticides and larvicides to reduce mosquito numbers (Connelly and Carlson, 2009).

Source reduction, the removal or covering of containers that hold water (e.g., tires, vases, water barrels), is useful in preventing anthropophilic container-breeding mosquitoes (e.g., *Aedes* spp.) from establishing populations near people. However, larvae in the mosquito genera *Culex* and *Anopheles* develop in open bodies of water, making water removal a less viable option for mosquito control. In habitats where source reduction is not possible (e.g., wetlands, roadside ditches, floodplains, ephemeral pools, irrigation channels, rice paddies), larvicides are commonly used to prevent adult mosquitoes from emerging. Larvicides include chemicals or modified pathogens developed specifically to kill larvae of a target pest species (Lawler, 2017). Common types of larvicides include Dipteran-killing bacteria, growth regulators (IGRs), surface films (SFs), and organophosphates (Connelly and Carlson, 2009; Mazzacano and Black,

2013). Insect Growth Regulators (IGRs) (e.g., methoprene or Altosid®) mimic juvenile fly hormones that regulate molting in larvae (Miura and Takahashi, 1973, 1974). These chemicals come in liquid and solid forms; liquids are sprayed on the surface of a target habitat, and solids are simply tossed in the water. Surface films (e.g., Agnique Monomolecular Film®) create a physical barrier between the water and atmosphere, preventing larvae and pupae from accessing atmospheric oxygen. Naturally occurring mosquito pathogens like *Bacillus thuringiensis israelensis* (*Bti*) and *Saccharopolyspora spinose* (spinosad) disrupt the targets' digestive system, which prevents them from obtaining enough nutrients for growth and molting. Finally, organophosphates (e.g., Temephos or Abate®) are pesticides that target the insect's nervous system, but the use of organophosphates is uncommon due to high toxicity to a wide variety of non-target vertebrate and invertebrate taxa (Sanchez-Bayo, 2012). All forms of larvicides have recommended dosages that can be calculated by approximating the surface area or volume of the body of water being treated. This thesis aims to answer questions relating to the non-target toxicity of IGRs and SFs, both of which are regularly used today.

For this project, non-target organisms will be defined as animals that are part of the natural aquatic community where mosquito larvae are found. Non-target aquatic insects are biologically and physically similar to mosquito larvae and may be more susceptible to growth hormones and SFs than other animal groups (e.g., crustaceans, amphibians, fish) (Lawler, 2017). For example, growth regulators mimic insect growth hormones necessary for ecdysis, and SFs may harm other insects like predatory beetle adults and their larvae that need access to atmospheric oxygen. The pathogen *Bti* is considered the most target specific larvicide used to control mosquito larvae, as it specifically affects flies from the

suborder Nematocera. Spinosad is used to control a wider variety of pest insects and has been shown to be lethal to non-biting chironomids (Duchet et al., 2015; Lawler, 2017). Insects that have been previously shown to exhibit lethal or sublethal effects, or reduction in abundance in response to these larvicides mentioned include predatory beetles (Order: Coleoptera), dragonflies and damselflies (Order: Odonata), mayflies (Order: Ephemeroptera), aquatic flies including non-target mosquitoes (Order: Diptera), stoneflies (Order: Plecoptera), and other non-insect invertebrates (Antwi and Reddy, 2015; Breaud et al., 1977; Lawler, 2017; Lawler and Dritz, 2013; Miura and Takahashi, 1973 and 1974; Norland and Mulla, 1975; Steelman and Schilling, 1972; Takahashi et al., 1984).

Mosquitoes and other small aquatic flies are quick to colonize and proliferate in small bodies of water (Chase and Knight, 2003; Walton et al., 1990). Thus, in a pond that has been treated or flushed, once lethal levels dissipate, aquatic flies will be one of the first insect groups to lay eggs, or hatch from previously laid diapausing eggs, and reestablish populations (Batzer and Wissinger, 1996). It likely takes larger animals, like predaceous diving beetles and dragonflies, longer to establish populations in newly inundated areas, as they often have longer generation times (Chase and Knight, 2003; Merritt et al., 2008).

Mosquito density in temporary wetland habitats can be lower than those in semi-permanent wetlands, due to predators and competitive organisms being well adapted to frequent drying and refilling in temporary habitats (Chase and Knight, 2003). Even though non-target predators are able to survive in intermittently drying habitats (Merritt et al., 2008; Strachan et al., 2015, Williams, 1996) the fact that that they have longer generation times than their prey means that if larvicides are directly lethal to these

predators, then it will take longer for their population to recover than mosquitoes once the habitat is flushed or dried, then refilled. Thus, understanding aquatic community structure as well as the underlying seasonal properties of a site before adding chemicals is important in predicting the effects of larvicides. Blanket spraying of all potential mosquito breeding sites could create an opportune window with lowered competitive or predaceous threats. Predator presence and availability of nutrients are two important factors that influence female mosquito oviposition preference and larval survival in a habitat (Blaustein et al., 2004 and 2005; Reiskind and Wilson, 2004; Reiskind et al, 2004; Stav et al., 2000; Vonesh, 2010). Flushing of a habitat can be potentially beneficial or harmful to mosquitoes, the net positive or negative effect of flushing depends on the type of habitat. For instance, Duchet et al. (2017) found that gravid females preferred to lay their eggs in unflushed mesocosms over flushed ones, which was likely due to the removal of larval nutrients after flushing. For the sites studied in this paper (i.e., roadside ditches), I would predict that flushing does not have a major effect on mosquito nutrient availability as rain events push detritus downstream as well as bring in new dead organic material from the surrounding landscape.

This idea of predatory release via non-target effects of pesticides benefitting pest species has been seen in agricultural pest control studies. Douglas et al. (2015) found that neonicotinoid application intended to kill insect pests also reduced predatory beetle numbers in soybean fields, which caused an increase in herbivorous slugs and lowered soybean yields. Although the mode of predatory release in the above example is different than my hypothesized scenario (slugs were not harmed by the neonicotinoids), this shows that chemicals can unintentionally lower predation rates and benefit the fitness of a pest

species. Martinou et al. (2014) learned that thiacloprid, a neonicotinoid, reduced predation rates of a predatory Hemipteran. Ahmad et al. (2003) found that ladybeetle larvae predation decreased after feeding on aphids exposed to neem oil, an organic pesticide used to control aphids. There are also examples of predatory release via agricultural pesticides benefitting mosquitoes. Dennett et al. (2003) conducted experiments examining the non-target effects of a pyrethroid insecticide (λ-cyhalothrin) used to control rice weevils. They determined that this chemical was more lethal to Notonectids (Order: Hemiptera) and Hydrophilids (Order: Coleoptera) than *Anopheles* mosquito larvae, suggesting that predator release via λ-cyhalothrin could be beneficial to *Anopheles* larvae. Grigarick et al. (1990) observed reductions in predatory insects caused by triphenyltin hydroxide, a fungicide used on rice fields, which resulted in a large recolonization of the mosquito larvae, *Culex tarsalis.*

In areas near people, tree hole mosquitoes (*Aedes* spp.) oviposit in manmade containers like discarded tires and flower vases. Open water mosquitoes (e.g., *Culex, Anopheles*) lay their eggs in ponds, wetlands, and ephemeral pools. In urban and rural areas, open water mosquito larvae can be commonly found in places like tire ruts, cattle troughs, and roadside ditches. Roadside ditches are dug for the purpose of diverting rainwater from the streets, which eventually drain into larger bodies of water like creeks, rivers, and lakes. Ruts and ditches like these are an excellent rearing site for many aquatic insect taxa because they are generally inundated during the wet season for long enough periods of time to complete their juvenile stage. Trees and tall grass are often directly above or close to these habitats and provide detritus like leaves as a food source, as well as shade during the day. In addition, they dry frequently enough to prevent small

insectivorous fish populations from establishing. Large aquatic insects like dragonflies, hemipterans, and beetles are the top predators in these systems, making these organisms important in limiting the abundance of prey insects like mosquitoes and other Diptera (Batzer and Wissinger, 1996).

This chapter aims to test the following hypotheses: 1) There will be differences in predator, prey, and whole community operational taxonomic unit (OTU) diversity metrics (Shannon's Diversity *(H')* richness *(S)*, evenness *(E)*, as well as total insect abundance and abundance within individual taxa groups) between pre-larvicide treatment and post-larvicide treatment samples from sites where SFs or IGRs have been added, and 2) There will not be differences in these metrics when comparing samples from untreated sites taken during the same time frame.

From the above hypotheses, I predicted that these metrics will be lower in post treatment samples after the introduction of IGRs and SFs. I also predicted that individual OTU groups will be affected differently depending on their biology. For example, aquatic Diptera will likely be negatively affected by IGRs, since they are physiologically and phylogenetically more similar to mosquitoes than other insect orders. Surface films will likely affect insects that need access to atmospheric oxygen (e.g., mosquito larvae/pupae, aquatic beetle adults, aquatic hemipterans), and insects with biological gills (e.g., odonate nymphs, mayfly larvae) or those adapted to hypoxic conditions (e.g., chironomid larvae) might not be harmed (Merritt et al., 2008). Previous studies have shown non-target insect communities may change in response to pesticide application, either through lethal effects of exposure, sublethal effects, or through food web alterations (Duchet et al., 2018; Hershey et al., 1998; Lawler, 2017; Miura and Takahashi, 1973 and 1974; Woin,

1998). Comparing control and untreated sites to those that contain SF and IGR larvicides will give insight into how these chemicals affect aquatic insect populations under real-world conditions. My prediction for the second hypothesis was that there will be no differences in these metrics among pre-treatment samples of my site groupings (i.e., no differences among SF, IGR, and control groups in terms of their initial samples). I predicted this because I expected these sites to be similar in community structure, as they were all in close proximity to each other.

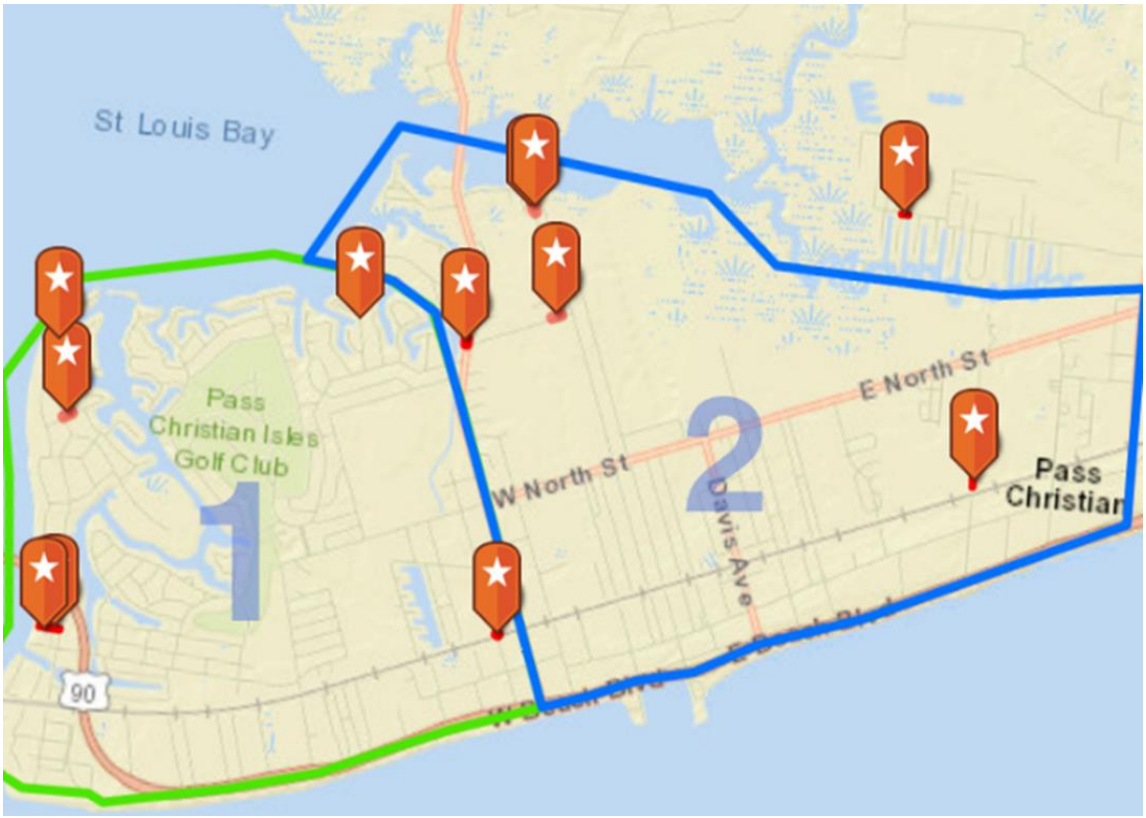

Fig. 1.1 Sampling locations in Harrison Co., Mississippi. Sections "1" and "2" are survey areas marked by Harrison Co. Mosquito Control. Northern most point is in survey area number 22, which is not marked on this map.

Fig. 1.2 Example of a roadside ditch habitat that was sampled.

Fig. 1.3 Half Square Meter Subsample. PVC square with aquatic D-net used for sampling

1.2 Materials and Methods

1.2.1 Site Characterization

Roadside ditches in coastal Harrison Co. were sampled before and after larvicide application, coordinated with the spraying maps of the Harrison Co. Mosquito Control Department during the summer and fall of 2018 (Figs. 1.1 and 1.2). Working with Harrison Co. Mosquito Control was beneficial to my field studies and experiments in numerous ways. First, Hattiesburg, MS was not a reliable survey area because Forrest

County does not have an official mosquito control department that oversees the treatment of mosquito breeding sites, and instead relies on various private pest control companies that work with the city to apply larvicides based on nuisance complaints made by residents. Second, Harrison Co. is the closest municipality to our location in Hattiesburg that has full oversight over larvicide spraying and adulticide fogging on public land and are also responsible for responding to residential nuisance complaints. They have a detailed map of every potential mosquito breeding site on public property (mainly roadside ditches), and routinely dip-survey these areas and treat them when they spot mosquito larvae. When a site is treated, they record the location and chemical used in a GIS program. These sites are treated with the appropriate chemical depending on the ratio of larvae to pupae.

I used their treatment records from past years and made note of areas that have been treated the most heavily to determine if there are any residual effects of larvicides on aquatic insect communities in this area. Although immediate residual effects in sites during this season will be known, all sites that I surveyed in Harrison Co. have most likely been treated at some point in the past due to the highly urbanized setting of my sample area. The community composition data from my surveys were also used for deciding which invertebrates to use in lab and field experiments for the second and third chapters of this thesis, as it was necessary to identify the most common organisms to simulate shallow wetlands and ditches that are treated for mosquitoes in southern Mississippi. In addition to Harrison Co., untreated sites were surveyed in rural areas around Hattiesburg. These untreated sites were used as the source of predatory specimens in my laboratory and field experiments.

1.2.2 Field Surveys and Identification

Samples in Harrison Co. were taken during the daytime immediately before and two weeks after the application of IGRs or SFs to assess the effects of these chemicals on insect communities. Prior to sampling, I checked each ditch for the presence of mosquitoes using a 250 mL mosquito dipper, a sampling device used by mosquito control professionals to calculate larval densities in a standardized way. Only sites that contained mosquito larvae or pupae were further sampled and later treated, ensuring that all sites included in this study were hospitable for mosquito larvae and other insects. Four sites were treated with IGRs and four were treated with SFs (Fig. 1.1). Treatment of IGR, SF, or control was chosen at random, using a random sequence generator website (random.org/sequences). Treatments were applied based on the recommended dosages found on the larvicides' labeling. The Altosid® IGR recommended rate was one briquette every 100 m^2, for a depth of 0-2 ft (no sites in this survey exceeded 2 ft in depth). The recommended Agnique® SF rate is 2-10 L per hectare in roadside ditches depending on level of pollution and vegetation, and the median (6 L per hectare) was chosen as the standard for all sites in this study. The amount of Agnique® used was calculated using the above rate and the surface area for each treated site. Four sites were purposely untreated to serve as water-only controls. Sampling equipment was either rinsed with fresh water or disposed of after sampling treated areas to avoid cross contamination of chemicals between sites.

Multiple 1 m^2 subsamples were taken equidistant from each other along the length of each site using an aquatic D-net. The number of subsamples depended on its approximate surface area, which was calculated from its length and width measured using a digital

measuring wheel. Larger sites had more 1 m^2 subsamples taken and were standardized as follows: ditches 1 - 25 m^2 = four subsamples, 25 - 100 m^2 = six subsamples, 100 - 500 m^2 = eight subsamples, and > 500 m^2 = 15 subsamples (based on sampling methods from similar sites in Pitcher and Yee (2018)). Samples were collected in a standardized way, by sampling the entire column of water within the 1 m^2 PVC square in up-and-down motions covering the entire square twice (Fig. 1.3). Contents inside the D-net were filtered using a 250-nanometer strainer and placed into 1000 mL Nalgene® bottles. In the field, a small amount of 95% ethanol was added to each container to kill specimens for later identification in the lab. Specimens were then preserved in 95% ethanol for long term storage. Insects collected from Harrison Co. sites were identified to lowest operational taxonomic unit (OTU), using identification manuals by Merritt et al. (2008), Epler (1996), and Wright and Peterson (1944). Taxa were also denoted as either predatory or prey groups based on ecological information from these publications. I chose to group specimens into lowest OTU groups because many specimens were early instars and had not yet developed the morphological characteristics necessary to identify them using published keys. For example, I collected numerous dragonfly (Suborder: Anisoptera) nymphs from several sites that had characteristics allowing me to identify them into two superfamilies: Aeshnoidea and Libelluloidea, but available taxonomic keys require late instar specimens for further identification (Merritt et al., 2008; Wright and Peterson, 1944). This was also the case for damselfly (Suborder: Zygoptera) nymphs and some aquatic beetle larvae (Order: Coleoptera). I grouped all predators by superfamily and prey insects (mostly Diptera larvae and pupae) by family, as most of my specimens were of late enough instar with intact bodies to accurately identify. Two

groups were identified to Order only: mayfly juveniles (Order: Ephemeroptera) and caddisfly larvae (Order: Trichoptera).

1.2.3 Sampling Effort Calculations

Using the site and predator/prey group abundance data, I constructed rarefaction curves rarefied to 50 individuals to account for highly abundant OTUs. These curves were created in R using the average of 10,000 permutations. Data was divided between the three-treatment levels (IGR, SF, and control) as I was only making comparisons between the before and after samples. To further evaluate my sampling effort, I estimated the total number of potential OTUs in my sample sites by calculating asymptotic diversity estimates using the iNEXT package in R, which generated an estimated value of possible species present by extrapolating my rarefied accumulation curves using the Chao 1 estimator (Chao et al., 2014; Hsieh, 2020). Then, I divided my observed richness by the estimated richness to obtain the proportion of collected species to potential total species in each grouping (groupings included all specimens sampled across all sites, IGR sites only, SF sites only, and control sites only).

1.2.4 *(H'), (S), (E),* and Abundance Calculation and Analysis

Species diversity metrics were compared between the combined values of all four replicate sites of the same treatment (IGR, SF, control). Species (OTU) richness *(S)*, is defined as the number of species per site or group of sites. Diversity was measured using the Shannon's index ($H' = -\sum_{i=1}^{S} p_i \ln p_i$) calculated from raw abundance data, as well as a measure of evenness ($E = \frac{H'}{\ln (S)}$), based on this metric (Shannon and Weaver, 1963). Shannon's diversity was used over other metrics because of its use in other studies relevant to my thesis (e.g., Marina, 2014; Pitcher and Yee, 2018; Thakare, 2011).

Furthermore, *(H')* is one of the most widely used diversity metrics in ecology, which allows for my results to be more easily compared to past and potential future studies similar to mine (Magurran, 1988).

Comparisons of the diversity and abundance metrics were made between pre- and post-treatment groups using paired two-tailed t-tests, with sites within treatments as replicates. I tested for assumptions of normality using a Shapiro-Wilk's goodness of fit test, and assumptions of homoscedasticity by plotting residual versus predicted values and checking for patterns. For data that was not normally distributed or had unequal variances, it was transformed to meet assumptions. All data met assumptions of homogeneous variances. If transformations were not possible and data remained non-normal, I conducted non-parametric Wilcoxon/Kruskal-Wallis Rank-Sums tests to determine if the before and after treatment levels were significantly different. Due to few replicates (< 40), χ^2 approximations generated by these Rank-Sum tests were evaluated. These metrics (diversity, richness, evenness, and abundance) were compared to help determine if IGR and SF application influenced diversity in these habitats. Analyses were done for the entire aquatic insect community collected (predators and prey insects combined), as well as predators and prey, separately.

1.2.5 ANOSIM

I performed four separate analyses of similarities (ANOSIM) examining similarity of OTU community structure between pre- and post-treatment samples of: all Harrison Co. samples, SF sites only, IGR sites only, and control sites only. An ANOSIM is an analysis technique that uses a dissimilarity matrix, instead of actual data, to determine differences among groups. In this case I used a Bray-Curtis dissimilarity calculation of all possible

pair-wise comparisons among samples (e.g., insect community proportions from site one pre-treatment sample to those proportions from site one post-treatment sample). Data used in each ANOSIM came from matrix rarefied to 50 individuals per replicate sample to account for highly abundant species. These rarefied datasets were then transformed into proportional abundances, and then Bray-Curtis dissimilarity indices were created using the *vegdist* function from the *vegan* package in R (Oksanen et al., 2019). The purpose of these ANOSIM tests is to determine if rank similarity differences within groups (pre-treatment replicates being compared to other pre-treatment replicates, and post-treatment replicates to other post-treatment replicates) being compared are significantly greater than rank similarity differences among groups (i.e., OTU differences among pre-treatment versus post-treatment replicates). If within differences are indeed greater than among differences, then the groups being compared (in this case pre- to post-larvicide treatment groups) are significantly different from each other. I also compared pre-treatment samples among the three treatment groups to determine if these sites were inherently different. The null hypothesis for these tests is that within differences are not greater than between differences for pre- and post-treatment communities in each treatment comparison. Analyses for all chapters in this these were conducted using JMP, SAS, and Rstudio statistical software (Kindt and Coe, 2005; R Core Team, 2019; SAS Institute, Inc., 2004, 2019). In the output, the ANOSIM Statistic R is the difference of mean ranks between groups being compared, on a scale of -1 to +1, where 0 = random. Significance < 0.05 means that rank similarity *among* data in within each group is significantly greater than rank similarity *between* data in groups being compared. Hereafter, all analyses use an alpha-value of 0.05 unless noted otherwise.

1.2.6 Changes in Individual OTU Abundances

The abundance of every recorded OTU between initial and after-treatment surveys was compared using the combined data of replicates from each treatment type (IGR, SF, and Control). Some OTU sample sizes were too low or were completely absent in all sites of a given treatment type and were not analyzed. For the OTUs that had enough individuals collected to make comparisons, the dataset of each group within each treatment type was tested for assumptions of normality and homoscedasticity. If a dataset did not meet these assumptions, they were transformed accordingly, or a non-parametric test was used instead.

1.3 Results

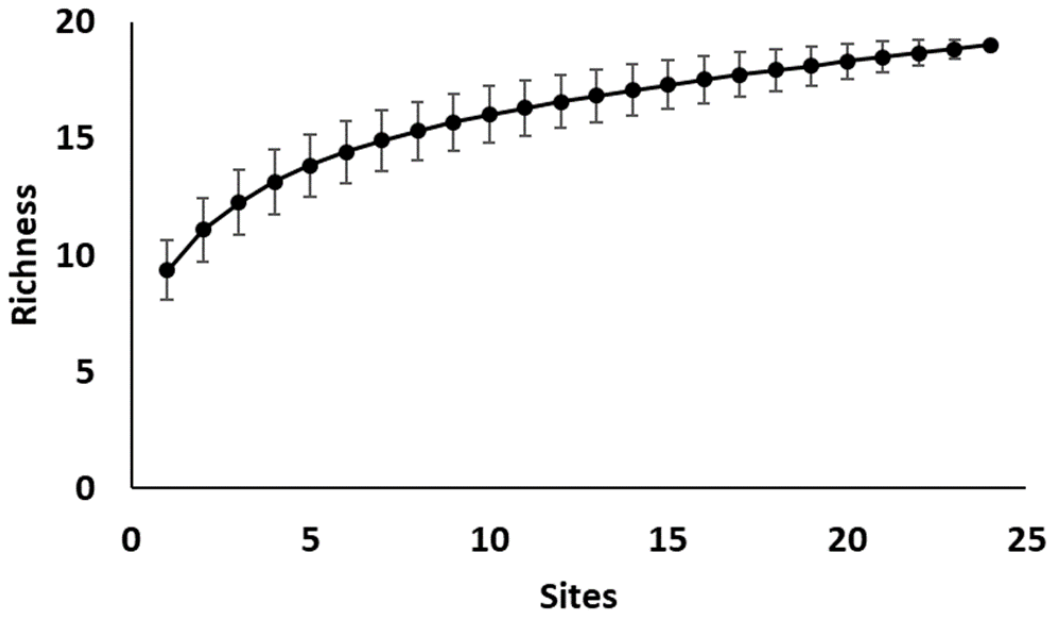

Fig. 1.4. Rarefied accumulation curve generated from average of 10,000 permutations of OTU abundance data collected from all treatment sites in Harrison Co. samples. X = number of sites, Y = number of unique OTUs (± 1 SD).

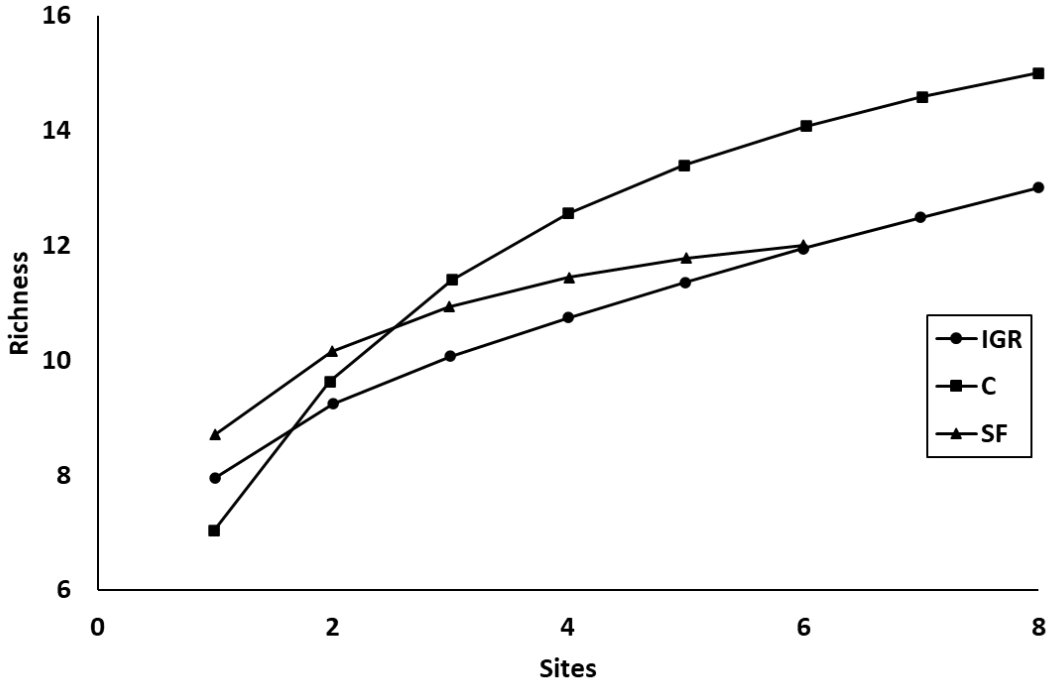

Fig. 1.5 Rarefied accumulation curves generated from average of 10,000 permutations of OTU abundance data, divided among growth regulator (IGR), control (C), and surface film (SF) treated sites in Harrison Co. X = number of sites, Y = number of unique OTUs (± 1 SD).

1.3.1 Sampling Effort

Rarefaction curves (Figures 1.4-1.5) that show the accumulated richness over the total number of sites sampled for each larvicide treatment type, generated from 10,000 permutations using a maximum of 50 random individuals in each permutation. All rarefaction curves leveled off but never reached their asymptote.

Table 1.1 Extrapolation Results to Assess Sampling Effort. Observed and estimated OTU richness (S) calculated from extrapolation of 5,000 sampled individuals using the Chao 1 estimator equation. Includes calculations of all habitat samples (Combined), SF treated sites, IGR treated sites, and control sites. "% Sampled" column = (Observed (S)/Estimator (S))*100), to give percentage of OTUs I collected to the total possible OTUs in each grouping

Grouping	Observed *(S)*	Estimated *(S)* ± 1 SE	% Sampled
Combined Sites *(S)*	19	26.996 ± 11.656	70.38
SF Sites *(S)*	13	20.995 ± 11.655	61.92
IGR Sites *(S)*	15	16.491 ± 2.280	90.96
Control Sites *(S)*	12	12.249 ± 0.727	97.98

After extrapolating rarefaction data, sites divided among treatments were not the same as all sites combined in terms of OTU richness (Table 1.1). This is important, as SF, IGR, and control treatment sites likely were all missing some OTUs that were found in the other two respective treatment groups (e.g., control sites had 12, but overall richness was 19). After calculations of total potential richness, overall sampling effort estimations show that I collected 70.38% of all possible OTUs in my sites.

Table 1.2 Habitat Survey Analyses. Results from two-tailed paired t-tests comparing before vs. after treatment changes in the metrics: Shannon's diversity (H'), Evenness (E), Richness (S), and total abundance (A). Treatments were: IGR, SF, and control (C). Comparisons conducted were between combined samples of sites of the same treatment, with individual sites as replicates. Data that needed to be transformed to meet assumptions of normality is labeled: Log = log transformed, SQRT = square root transformed, or no = no transformation. Significant values are in bold.

Grouping	Transformed	Metric	Treatment	Difference (pre- vs. post-treatment means) ± 1 SE	t	df	p-value
Community	No	*(H')*	IGR	-0.346 ± 0.289	-1.194	3	0.318
	No	*(H')*	C	-0.757 ± 0.456	-1.661	3	0.195
	No	*(H')*	SF	0.287 ± 0.230	1.245	2	0.339
	No	*(S)*	IGR	-1.000 ± 1.000	-1.000	3	0.391
	No	*(S)*	C	-3.500 ± 1.848	-1.894	3	0.155
	SQRT	*(S)*	SF	-0.304 ± 0.304	-1.000	2	0.423
	No	*(E)*	IGR	-0.115 ± 0.178	-0.650	3	0.562
	No	*(E)*	C	-0.411 ± 0.315	-1.304	3	0.283
	No	*(E)*	SF	0.282 ± 0.061	4.606	2	**0.044**
	No	*A*	C	-6.000 ± 9.857	-0.609	3	0.586
	No	*A*	SF	-33.000 ± 10.692	-3.086	2	0.091
Predators Only	No	*(H')*	IGR	-0.163 ± 0.125	-1.303	3	0.284
	No	*(H')*	C	-0.198 ± 0.258	-0.767	3	0.500
	No	*(H')*	SF	0.080 ± 0.455	0.177	3	0.871
	No	*(S)*	IGR	-0.500 ± 0.866	-0.577	3	0.604
	No	*(S)*	C	-1.000 ± 1.080	-0.926	3	0.423
	No	*(S)*	SF	-0.250 ± 1.108	-0.225	3	0.836
	No	*(E)*	IGR	0.058 ± 0.171	0.343	3	0.754
	No	*(E)*	C	-0.156 ± 0.283	-0.547	3	0.622
	No	*(E)*	SF	0.015 ± 0.287	0.055	3	0.960

Table 1.2 Continued

	No	*A*	IGR	25.500 ± 21.730	1.173	3	0.325
	No	*A*	C	-3.750 ± 8.159	-0.460	3	0.677
	Log	*A*	SF	-0.722 ± 0.411	-1.758	3	0.177
Prey Only	Log	*(H')*	IGR	-0.405 ± 0.304	-1.335	3	0.274
	No	*(H')*	C	-0.771 ± 0.348	-2.214	3	0.114
	No	*(H')*	SF	0.060 ± 0.370	0.164	2	0.885
	No	*(S)*	IGR	-0.500 ± 0.866	-0.577	3	0.604
	No	*(S)*	C	-2.500 ± 0.866	-2.887	3	**0.032**
	No	*(S)*	SF	-0.333 ± 0.667	-0.500	2	0.667
	No	*(E)*	C	-0.666 ± 0.281	-2.374	3	**0.049**
	No	*(E)*	SF	0.302 ± 0.406	0.744	2	0.534
	Log	*A*	IGR	0.957 ± 1.766	0.542	2	0.642
	SQRT	*A*	C	-1.202 ± 0.565	-2.127	3	0.123
	No	*A*	SF	-7.333 ± 10.477	-0.700	2	0.556

Table 1.3 Non-parametric Habitat Survey Analyses. Results from non-parametric Wilcoxon/Kruskal-Wallis Rank-Sum tests (using χ^2 approximation) comparing before vs. after treatment changes in the metrics: Evenness *(E)* and total abundance *(A)*. Only two groupings from growth regulator (IGR) treated sites needed to be analyzed using a non-parametric test. Comparisons conducted were between combined samples of sites of the same treatment, with individual sites as replicates.

Grouping	Metric	Treatment	χ^2	DF	Prob > χ^2
Community	*(A)*	IGR	1.333	1	0.248
Prey Only	*(E)*	IGR	0.094	1	0.758

1.3.2 *(H')*, *(S)*, *(E)*, and Abundance

For the predators and prey combined (community) there was a significant difference in evenness when comparing before and after treatments of SFs (Table 1.2). Specifically, evenness was lower after application of SFs. There were no significant differences when only comparing predators within treatments. For prey only comparisons, there was a significant difference in *H'* and evenness between before and after samples of controls (Table 1.2), with greater *H'* and evenness after the application than before. From the non-parametric tests that were performed, no treatments were shown to be unequal after the two-week sample period (Table 1.3).

Table 1.4 Community Change by Treatment: ANOSIM. Results from Analysis of Similarity (ANOSIM) (5,000 permutations of samples rarefied to 50 individuals) comparing rank similarity (rank data generated from OTU abundance data) between pre- and post-treatment samples within all combined sites, surface film (SF) sites only, growth regulator (IGR) sites only, and control sites only.

Grouping	ANOSIM Statistic R	Significance
Combined Sites	-0.040	0.674
SF Sites	-0.593	1.000
IGR Sites	-0.130	0.609
Control Sites	-0.179	0.667

Table 1.5 Similarity between initial samples: ANOSIM. Results from Analysis of Similarity (ANOSIM) (5,000 permutations of samples rarefied to 50 individuals) comparing rank similarity (rank data generated from OTU abundance data) of pre-treatment communities between pairs of each treatment type (surface films = SF, growth regulator = IGR, and control).

Grouping	ANOSIM Statistic R	Significance
IGR vs SF	-0.099	0.662
Control vs SF	-0.036	0.600
Control vs IGR	-0.018	0.467

1.3.3 ANOSIM

There were no significant differences in communities between pre-and post-treatment groups under all four comparisons (Table 1.4). After conducting another ANOSIM comparing community compositions of pre-treatment samples among SF sites, IGR sites, and control sites, there were no significant differences (Table 1.5).

Table 1.6 Individual OTU Abundance. Abundance data of every Operational Taxonomic Unit (OTU) from all replicate samples of sites separated by treatment type. IGR (n=4), SF (n=3), and C (n=4). "Before" = total number of individuals collected from all replicates before treatment, "After" = total number of individuals taken two weeks after treatment.

	IGR		SF		C	
OTU Group	Before	After	Before	After	Before	After
Hydrophiloidea	15	20	0	2	13	31
Dytiscoidea	4	13	5	20	1	4
Coenagrionoidea	22	9	14	2	29	14
Libelulloidea	128	19	4	5	1	5
Aeshnoidea	1	0	1	82	0	4
Nepoidea	0	7	15	6	0	1
Corixoidea	0	0	1	0	0	0
Bhyrroidea	0	0	0	0	0	1
Chironomidae	669	580	6	36	19	24
Culicidae	72	9	21	9	1	3
Ephemeroptera	10	0	0	0	0	0
Ceratopogonidae	1	1	0	5	0	2
Trichoptera	0	0	0	0	3	2
Stratiomyidae	0	1	0	0	4	3
Sciomyzidae	0	0	4	1	2	0
Tipulidae	0	0	0	0	0	1
Ephydridae (pupae)	0	0	0	2	0	0
Psychodidae	0	1	0	0	0	0
Dolichopodidae	0	1	0	0	0	2

Table 1.7 Individual OTU Analyses (Parametric). Parametric tests (two-tailed paired t-tests) on OTU abundance data that achieved normal distribution after transformation, comparing mean number of individuals per OTU group collected before treatment (Before) to mean number of individuals after treatment (After) from each treatment type (growth regulator = IGR, surface films = SF and Control = C).

OTU Group	Treatment	Transformation	Before	After	SE	p
Hydrophiloidea	IGR	SQRT	1.559	1.683	± 0.315	0.720
Dytiscoidea	SF	SQRT	1.000	2.081	± 0.649	0.238
Coenagrionoidea	C	SQRT	1.811	1.500	± 0.580	0.629
Libelulloidea	IGR	SQRT	4.502	1.604	± 2.388	0.312
Nepoidea	SF	SQRT	1.799	1.079	± 0.939	0.523
Chironomidae	SF	Log	0.598	1.607	± 0.870	0.366
Culicidae	SF	SQRT	1.824	1.000	± 0.434	0.198

Table 1.8 Individual OTU Analyses (non-parametric). Wilcoxon/Kruskal-Wallis Rank-Sums tests comparing pre-treatment to post-treatment values on un-transformable individual OTU data. NA = OTU was not present in any replicate of a given treatment. "Notes" column indicates the number of individuals that were collected when a given OTU was present in only one suite of replicate samples (before treatment = B, or after treatment A) and absent in the other. All tests had one degree of freedom.

OTU Group	Treatment	χ^2	Prob > χ^2	Notes
Hydrophiloidea	SF	2.5000	0.1138	
Dytiscoidea	IGR	0.4375	0.8770	
	C	1.0000	0.3173	1 B, 4 A
Coenagrionoidea	IGR	0.0240	0.1913	
	SF	0.0556	0.8137	
Libelulloidea	SF	0.0667	0.7963	
	C	0.6944	0.4047	
Aeshnoidea	IGR	1.0000	0.3173	1 B, 0 A
	SF	0.0667	0.7963	
	C	1.0000	0.3173	0 B, 4 A
Nepoidea	IGR	2.2857	0.1306	0 B, 7 A
	C	1.0000	0.3173	0 B, 1 A
Corixoidea	IGR	NA	NA	0,0
	SF	1.0000	0.3173	1 B, 0 A
	C	NA	NA	0,0
Bhyrroidea	IGR	NA	NA	0,0
	SF	NA	NA	0,0
	C	1.0000	0.3173	0 B, 1 A
Chironomidae	IGR	0.0476	0.8273	
	C	1.8173	0.1776	
Culicidae	IGR	0.4375	0.5083	
	C	0.6944	0.4047	
Ephemeroptera	IGR	1.0000	0.3173	10 B, 0 A
	SF	NA	NA	0,0
	C	NA	NA	0,0
Ceratopogonidae	IGR	NA	NA	1,1
	SF	1.0000	0.3173	0 B, 5A
	C	2.3333	0.1266	0 B, 2 A
Trichoptera	IGR	NA	NA	0,0
	SF	NA	NA	0,0
	C	0.1111	0.7389	
Stratiomyidae	IGR	1.0000	0.3173	0 B, 1 A

Table 1.8 Continued

	SF	NA	NA	0,0
	C	0.0357	0.8501	
Sciomyzidae	IGR	NA	NA	0,0
	SF	0.0667	0.7963	
	C	1.0000	0.3173	2 B, 0 A
Tipulidae	IGR	NA	NA	0,0
	SF	NA	NA	0,0
	C	1.0000	0.3173	0 B, 1 A
Ephydridae (pupae)	IGR	NA	NA	0,0
	SF	1.0000	0.3173	0 B, 2 A
	C	NA	NA	0,0
Psychodidae	IGR	1	0.3173	0 B, 1 A
	SF	NA	NA	0,0
	C	NA	NA	0,0
Dolichopodidae	IGR	1	0.3173	0 B, 1 A
	SF	NA	NA	0,0
	C	2.3333	0.1266	0 B, 2 A

1.3.4 Changes in Individual OTU Abundances

Insects collected from Harrison Co. were identified and placed in 19 OTU groups (Table 1.6). For the two-tailed paired t-tests conducted on OTUs whose abundance data met assumptions of normality of transformation, there were no significant differences between the initial and post-treatment samples (Table 1.7). For OTU data that was non-normal even after transformations, non-parametric Wilcoxon/Kruskal-Wallis Rank-Sum tests found no significant differences in OTU abundances between before and after samples for each respective treatment type (Table 1.8).

1.4 Discussion

Calculations of sample effort from accumulation curves and the Chao 1 estimator indicate that sampling effort could have been improved. The estimated percentage of OTUs collected from all combined sites was 70.38. The proportion of sampled richness and estimated richness varied among treatment groups (IGRs, SFs, and control replicates), which I believe is due to small sample sizes. Lower richness for individual treatment group replicates was expected as I reduced the sample size to 1/3 of the entire dataset. One of the vials containing SF site data had cracked, causing ethanol to evaporate. The specimens in this vial became moldy, making it impossible to identify them. This loss in data is likely a partial cause for the small percentage of observed to estimated richness seen in SF treatments (61.92%), compared to IGR (90.96%) and control (97.98%) site data.

Results from diversity and abundance analyses suggest that there were no significant changes in insect communities caused by larvicide application. From the first set of ANOSIM tests examining pre-treatment to post-treatment sites within each treatment

type (SF, IGR, control), rank similarity *among* community composition data within before and after-treatment sites was not significantly greater than rank similarity *between* before and after-treatment sites. This suggests that there were no significant differences in OTU community composition before and after chemical applications. As predicted, I did not find significant differences in community composition among pre-treatment samples of my three treatment groups using an ANOSIM. With these results in mind, I still compared my three treatment groups (SF, IGR, control) separately, when examining community structure change after larvicide addition. This was done because the larvicides I used likely affect organisms through different modes of action and may affect different taxa and life stages (e.g., growth hormone regulation versus suffocation).

Prey and predator taxa were denoted as such based on their known ecology. Prey in this study included insects that primarily filter feed, eat dead organic matter, biofilms, or plant material. Prey taxa consisted of aquatic fly larvae and pupae (Order: Diptera), mayfly larvae (Order: Ephemeroptera), Caddisfly larvae (Order: Trichoptera), and riffle beetle larvae (Coleoptera: Elmidae). Predatory groups consisted of aquatic beetle larvae and adults (Coleoptera: Dytiscidae, Hydrophilidae, Noteridae), dragonflies and damselflies (Order: Odonata), and aquatic bugs (Hemiptera: Belostomatidae and Corixidae) (Table 1.8).

It is difficult to compare the community assemblages of aquatic insects found in this thesis to past work, as there is not much literature that focuses on roadside ditches in the southern United States. The most similar study to mine in terms of geographical relevance and methods was conducted by Pitcher and Yee (2018). While this study sampled roadside ditches in southern Mississippi, it only focused on aquatic beetle

diversity. However, comparisons can still be made if I use the genera-level aquatic beetle data from my sites. Pitcher and Yee's May-June highway ditch samples had an average adult richness of 2.44 per site, and an average larval richness = 0.66. August-October samples from Pitcher and Yee had an average adult richness of 2.18, and an average larval richness of 0.73. My sites, which include samples before and after treatment that were sampled between July and October had an average adult richness of = 1.29, and larval richness of 1.00 per site. While the study by Pitcher and Yee (2018) had similar collection methods as mine, they sampled a wider variety of site types and sizes (I only included their roadside data, but ponds and tire ruts were sampled as well). Also, some of the roadside ditches they sampled had a much larger surface area than those in this thesis (Pitcher and Yee sampled ditches on long highway stretches, and mine were more residential). Also, all of my samples were taken in coastal Mississippi within an area of about 12 square km, and the samples taken by Pitcher and Yee were from sites up to 40 km from Hattiesburg MS. These site differences in site type, geographical location, and area scope might be the reason for differences in larval and adult beetle richness between these two studies.

I hypothesized that changes in insect communities would be seen when comparing pre-larvicide treatment sites to post-larvicide treatment sites, and no changes would be seen when comparing insect communities from control sites (the only difference samples one and two in control sites are factors beyond my control like time and precipitation). The only significant differences were seen when comparing community evenness in SFs, prey diversity in control treatments, and prey evenness in control treatments (Table 1.2). For the comparison of changes in whole community evenness within treatments of SFs,

pre-treatment sites had higher mean evenness than post-treatment sites, indicating that the number of individuals collected from pre-treatment sites were more evenly spread across all OTUs than post-treatment sites. This might suggest that in the presence of SFs, tolerance of this chemical might differ among species. However, prey diversity and evenness significantly changed in control sites. This, coupled with the fact that no predator and prey metrics were significantly different in SF or IGR treatments, suggests that even though evenness changed in SF treated communities, chemical applications likely did not play a large role in shaping these communities. A study by Butler et al. (2010) found similar results of no negative community effects after the addition of methoprene to aquatic communities. While Butler et al. (2010) used different sampling methods, in different habitat types, and in a different region than this study, this example helps support my findings. In field tests, Miura and Takashi (1973) also found little negative effects of methoprene on non-target organisms, except for some aquatic Diptera.

In addition to community differences, I examined changes in each OTU after the addition of SFs, IGRs, and between samples one and two of control replicates. I found no significant differences in the abundance of any OTU within treatment types, but reducing the community abundance data down to these individual groups resulted in very low sample sizes, making it difficult to detect changes that might be caused by chemical treatment. While there were no statistically significant changes, there are some distinct differences in abundance for some groups (Table 1.6). For example (and as expected), there was a trend of mosquito numbers being higher in pre-treatment SF and IGR samples than post-treatment samples. In addition to this, chironomid numbers were higher in post-treatment SF sites their respective pre-treatment sites and lower in pre-treatment IGR

sites compared to post-treatment IGR sites. This may suggest that IGRs were the cause for lower chironomid numbers, however this result has already been documented (Breaud et al., 1977; Hershey et al., 1998; Norland and Mulla, 1975). Plus, IGR products like Strike® are used to control for biting midges (Diptera: Ceratopogonidae, which are in the same superfamily as Chironomidae) and contain S-methoprene, the same active ingredient as Altosid® (the product used in this study). The fact that chironomid numbers did not decline after the introduction of SFs, might be due to their habitat preference (the benthos) and mode of obtaining oxygen (cutaneous respiration) (Cooper et al., 2009; Merritt et al, 2008). However, these are only speculations since no statistical differences were found. Lastly, there were also notable changes in individual odonate groups (e.g., 1 aeshnid in pre-treatment SF sites vs. 82 post-treatment, 128 libellulids in pre-treatment IGR sites vs. 19 post treatment), which were the result of many hatchling-sized nymphs being collected from a single site within that treatment group.

I may not have found many community effects after the introduction of SFs and IGRs for several reasons. These field tests were conducted over a short time frame (two weeks) in sites that were desiccating and refilling. The 12 sites that were included in this analysis were those that were inundated during the initial pre-treatment sampling and were at a relatively similar depth and surface area two-weeks later. There were multiple sites that I had sampled and treated initially but were completely dry or only a small fraction of the original surface area when I came back to re-sample them. These sites that had drastically changed were not re-sampled. Because the samples I included were two weeks apart, I cannot confidently say that they remained at these depths for the entire two weeks when I was not there. The sites I sampled may be low in insect diversity overall, and the fact that

they are constantly inundating and drying might be the determining factor of richness and abundance of invertebrates, regardless of the addition of larvicides. It is also possible that changes in diversity were not detected even if there were lethal effects of these two larvicides on certain taxa, because if the systems I sampled have such low diversity, abundance, and richness to begin with, the composition of new insects returning to a treated system might look similar to the site before it was disturbed. Permanence of a water body has been shown to be one of the main determining components in species richness, with longer inundation periods leading to higher species richness (Wellborn et al., 1996). In addition to drying, there are many other factors that determine what insects can survive in a temporary pool, which ultimately determine the community structure of sites like roadside ditches and small ponds (Williams, 1996). It is also possible that the recommended dosages used to treat sites during this study were too low to detect effects in the insect community. For example, all SF and IGR site dosages a standardized amount per surface area or volume, and other factors that may impact a chemical's efficacy (e.g., pollution, vegetation, sunlight exposure, flow rate) were not accounted measured for each individual site.

In aquatic ecosystems, there is an equilibrium of input and output of nutrients and energy in the form of living and dead biota, microbial films on detritus, as well as inorganic compounds (Merritt et al., 2008). Food energy equilibrium is important in maintaining the natural densities and existence of endemic species. Introducing chemicals designed to kill larval insects may cause an imbalance of the food web by harming top predator insects like dragonflies and predaceous diving beetles, as well as invertebrate herbivores (Hershey et al., 1998). In a study by Duchet et al. (2018) researchers examined

invertebrate community changes after the introduction of different larvicides (larvicides in this study were *Bti*, organophosphates, and pyriproxyfen IGR). They found that pyriproxyfen reduced the abundance of mosquito larvae and their filter feeding competitors, which caused an increase in algae which then became more attractive to adult female mosquitoes as an oviposition site. However, mosquito larvae survival was lowest in these IGR treated habitats as lethal levels had not yet dissipated. Knowing that insects are a major energy source in aquatic and terrestrial food webs, negative bottom-up effects caused by larvicides could affect entire communities of organisms (e.g., the removal of Chironomids as a food source for predatory insects) (Hershey et al., 1998). Pyriproxyfen is less commonly used than methoprene due to its non-target toxicity to a wider range of organisms (Lawler, 2017).

The above examples show that the more information gained about the safety of applying larvicides like these to aquatic habitats, the better equipped we will be in our efforts to control mosquito populations. To gain a better understanding of how larvicides affect aquatic communities; larger sample areas, a wider variety of site types (not just roadside ditches), and longer survey periods could be conducted in the future. Assessing sites treated with other larvicides (e.g., *Bti*, spinosad) would help to further determine the impacts that these chemicals have on invertebrate communities. Studying factors that limit mosquito abundance and dispersal will help improve future mosquito control practices and limit the transmission of diseases associated with mosquito species. Additional research needs to be conducted in order to learn about complex interactions that occur in sites that are treated with larvicides. Using natural predators alongside other integrated mosquito management methods could be a promising strategy for mosquito

control, like it has been in agriculture. Culler and Lamp (2009) found that larval Dytiscids (Genus: *Agabus*) preferred to prey on mosquito larvae over other invertebrate prey, which is a great example of how native predaceous insects could be useful for mosquito suppression efforts. Insect growth regulators like methoprene stop dipterans from molting and may affect non-mosquito dipterans and other insects either directly, or via trophic interactions by reducing prey insect numbers (Hershey et al., 1998; Norland and Mulla, 1975). Other past research, however, has found no significant negative effects, or only short-term changes on non-target aquatic insect and arthropod communities after the addition of *Bti* or insect growth regulators (Davis and Peterson 2008; Russell et al., 2009). Conflicting results like these, suggest that the non-target effects of application of larvicides like IGRs is dependent on the pre-existing abiotic and biotic properties of the site being treated. Even the intended larvicidal effectiveness of *Bti* on target mosquitoes is dependent on many different environmental factors (Boisvert and Boisvert, 2000; Lacey, 2007). It is also important to note that this thesis only focused on aquatic insect communities. There are many more organisms common in roadside ditches that interact with mosqutio larvae (e.g., crustaceans, spiders, worms, snails, amphibians), that I did not sample to assess the effects of SFs and IGRs. Thus, conducting surveys like mine that collect a wider variety of taxa after treatment will provide a more accurate picture of how larvicides affect communities in ephemeral habitats.

CHAPTER II: EFFECTS OF LARVICIDES ON PREDATOR BEHAVIOR

2.1 Introduction

Current mosquito control practice uses an integrated approach to lower adult populations and the pathogens they vector. Surveillance, source reduction, and public education are all essential components of integrated mosquito management (Mazzacano and Black, 2013). While source reduction is the most effective method for controlling container-dwelling mosquitoes (i.e., *Aedes* spp.) the use of larvicides may be necessary to prevent adults from emerging from habitats that cannot be drained. For example, other medically important mosquito genera like *Culex* and *Anopheles* lay their eggs on the water's surface, generally in larger bodies of water like ponds, roadside ditches, wetlands, and tire ruts (Clements, 1999). Floodwater mosquitoes like *Psorophora* and some species of *Aedes* lay their eggs on soil in anticipation of a flood event and emerge in large numbers in large pools created by heavy rain, or from newly flooded irrigation ditches (Gouge et al., 2016). Mosquito control professionals face several challenges when deciding when and where to apply certain chemicals. If an aquatic habitat is on private property (e.g., discarded tires, septic systems, garbage, ponds), they first need permission from the owner to survey, drain, or treat these habitats. While they do not need permission to fog an area with adulticides in a neighborhood, this method only kills adult mosquitoes, leaving the source population of larvae unharmed.

While larvicides are effective at reducing mosquitoes at their source, using a single chemical too frequently will cause a buildup of resistance in the local pest's population (Connelly and Carlson, 2009). Biological control using natural enemies of mosquito larvae is a relatively new approach in public health entomology (it is a far more prevalent

practice in agricultural pest control). Mosquitofish (*Gambusia affinis*) are one of the more commonly used organisms implemented in mosquito IPM practices. However, fish are only effective as long as the habitat is inundated with water. In contrast, insects have evolved to take advantage ephemeral habitats in order to avoid large vertebrate predators like fish (e.g., fully aquatic adults can fly and disperse, some taxa lay desiccation-resistant eggs, aquatic larvae mature quickly or burrow and aestivate) (Merritt et al., 2008; Strachan et al., 2015, Williams, 1996). Thus, a more effective organism to use in biological control is one that occupies the same ecological niche as mosquito larvae and can withstand the same environmental pressures. Predatory copepods have been implemented in biological control, as they prey on early instar mosquito larvae (Brown et al., 1991; Marten et al., 1994). However, while there are Copepod species that eat mosquito larvae and are adapted to frequent desiccation in temporary pools, they are not effective enough to be considered as a biological control. The Copepod species that are effective at lowering mosquito numbers cannot survive in habitats that completely dry and need to be mass produced and reintroduced when a site is refilled with water (Marten et al., 1994). Aquatic insects like adult and larval beetles, hemipterans, predatory fly larvae, and odonates inhabit temporary habitats and prey on aquatic diptera like mosquitoes. Without communities of these predaceous invertebrates that regularly consume mosquito larvae, mosquito populations would likely be higher in ephemeral pools (Connelly and Carlson, 2009; Kumar and Jiang-Shiou, 2006; Mogi, 2007; Shaalan and Canyon, 2009).

Negative effects of larvicides have been observed on non-target aquatic insects that are known to prey on mosquito larvae (e.g., aquatic beetles, hemipterans, odonates)

(Antwi and Reddy, 2015; Breaud et al., 1977; Lawler, 2017; Miles et al., 2002; Miura and Takahashi, 1973 and 1974; Norland and Mulla, 1975; Steelman and Schilling, 1972; Takahashi et al., 1984). Therefore, unintentionally harming non-target predators that exist in treated habitats might be beneficial for future mosquito populations, as these taxa have longer generation times that small aquatic Diptera (Chase and Knight, 2003; Merritt et al., 2008). Even if these chemicals are not directly lethal to non-target predators, there may be sublethal behavioral effects that negatively influence their locomotion and hunting behavior. These sublethal effects may in turn, reduce the effectiveness of these predators at regulating larval mosquito numbers.

It is important to know how a chemical may change the way an organism moves or finds its food, but the few studies that have investigated behavioral responses of predaceous insects to pesticides have only been conducted in agricultural settings. Cox and Wilson (1984) found that honeybee workers exposed to the insecticide permethrin spent less time foraging and feeding, and more time cleaning their bodies than untreated bees. Research by Claver et al. (2003) showed that the hunting ability of a non-target predatory Hemipteran (*Acanthaspis pedestris*) was lowered by the pyrethroid cypermethrin. Kunkel et al. (2001) studied predatory ground beetles (Family: Carabidae) in turfgrass systems and observed weakened mobility and increased grooming activity when exposed to imidacloprid, a neonicotinoid. Another study found that deltamethrin (pyrethroid) application caused predaceous ladybird beetles (*Coccinella septempunctata*) to move and groom more often, as well as occupy different locations within crops (Wiles and Jepson, 1994). De Jiu and Waage (1990) found that parasitoid wasp foraging

behavior changed when exposed to permethrin (pyrethroid) and malathion (organophosphate).

From the above examples of research that described sublethal effects of pesticides on non-target organisms (Claver et al., 2003; Cox and Wilson, 1984; Jiu and Waage, 1990; Kunkel et al., 2001; Wiles and Jepson, 1994), as well as research that has found negative population-level effects of Insect Growth Regulators (IGRs) and Surface Films (SFs) on non-target aquatic insects (Antwi and Reddy, 2015; Breaud et al., 1977; Lawler, 2017; Miura and Takahashi, 1973 and 1974; Norland and Mulla, 1975; Steelman and Schilling, 1972; Takahashi et al., 1984), I hypothesized that when exposed to larvicides, non-target aquatic mosquito predators will exhibit behaviors different to those in treatments containing no larvicides. To address my hypothesis, I conducted laboratory observations examining larvicidal effects on common predatory taxa known to prey on mosquito larvae (Culler and Lamp, 2009; Floore et al., 2007; Kumar and Jiang-Shiou, 2006; Shaalan and Canyon, 2009). These predatory groups include *Laccophilus* adults and larvae (Coleoptera: Dytiscidae), dragonfly nymphs (Odonata: Libellulidae), and damselfly nymphs (Odonata: Coenagrionidae). I tested for behavioral changes in these taxa after exposing them to larvicides. I also examined the number of mosquito larvae eaten among different larvicide types and concentrations within each predator group during behavioral trials. For this, I hypothesized that prey consumption will vary among predators exposed to different treatments. While I could not find any previous literature examining prey consumption of aquatic insect predators after exposure to larvicides, there has been some research done showcasing negative effects of crop insecticides on the hunting ability of beneficial agricultural predators (Ahmad et al., 2003; Martinou et

al., 2014). Once behavioral experiments were completed, daily mortality over one week was recorded to assess potential long-term effects of larvicide treatments on survival. I hypothesized that survival rates within each of my four predatory groups would differ based on larvicide type and concentration. The above hypotheses for behavior, survival, and prey consumption are based on the known biology of these organisms. Specifically, I predicted that diving beetle adults and larvae would be more negatively affected by SFs than the odonates since they need to access the atmosphere for oxygen (Merritt et al., 2008), and that surface films would harm aquatic beetles in the same way they are intended to kill mosquito larvae and pupae. Odonates obtain their oxygen using gills (Merritt et al., 2008). Thus, I predicted that dragonfly and damselfly nymphs exposed to any concentration of SF would not differ from their control-treated counterparts in behavior, mortality, and number of mosquito larvae eaten. In terms of SF concentration, I predicted that the higher concentrations would show more negative effects on aquatic beetles than lower concentrations. Surface films are hydrophobic and stay on the water's surface. Therefore, when more of a surface film or any kind of oil is added to a body of water, the thicker the layer gets (or the larger the surface area it can effectively cover): increasing the amount of substance that interferes with their ability to utilize the water's surface tension. Past research has shown negative population-level effects of SFs on atmospheric-breathing aquatic insects like Corixids, Notonectids, and *Tropisternus lateralis* (Coleoptera: Hydrophilidae) adults (Takahashi, et al, 1984). This study by Takahashi et al. (1984) found no significant differences in dytiscid numbers, but they also mention that sample sizes of this family were too small to discern differences between treatments, and that some dead dytiscids were found in traps placed in habitats treated

with SFs. I predict that IGRs would most negatively affect diving beetle larvae, since IGRs are designed to halt molting in holometabolous insects (e.g., Diptera, Lepidoptera, Coleoptera). This was my prediction since beetles share more phylogenetically conserved traits to flies than odonates (beetles and flies both are fully metamorphic), thus, beetle larvae will be more negatively affected than odonates (Merritt et al., 2008). Past work by Steelman and Schilling (1972) and Norland and Mulla (1975) found the abundance of dytiscid larvae to be significantly lower in habitats treated with IGRs compared to control habitats. In the same study by Norland and Mulla (1975), they measured dragonfly and damselfly abundance after IGR applications and found no negative effects. Also, because beetles no longer molt once they reach the adult stage, I predicted that aquatic beetle adults would not show any negative effects after being exposed to any IGR concentration. The purpose of every observation in this study was necessary for determining which, and to what degree, of these common non-target mosquito predators are affected by the larvicides in question. While past studies have examined lethal concentrations and changes in abundance of non-target insects after larvicide exposure (Antwi and Reddy, 2015; Breaud et al., 1977; Lawler, 2017; Miura and Takahashi, 1973 and 1974; Norland and Mulla, 1975; Steelman and Schilling, 1972; Takahashi et al., 1984), this is the first to directly examine changes in hunting and locomotion behavior of non-targets after being exposed to SFs and IGRs.

2.2 Materials and Methods

Fig. 2.1 Example of a source of predators used in experiments in Chapters II and III. A tire rut filled with water near Lake Thoreau in Hattiesburg, MS.

2.2.1 Collection and Treatments

Swimming and hunting behavior of predatory insects were observed under varying concentrations of IGRs and SFs. Predators in these experiments were collected from untreated sites around Hattiesburg, MS (Lake Sehoy: 31.352768°N, -89.362825°W, Lake Thoreau Environmental Center (LTEC): 31.368154°N, -89.432707°W, and Petal River Park: 31.342412°N, 89.275838°W) one week prior to experiment start (Fig. 2.1). Individuals were kept alive at the University of Southern Mississippi (USM) campus in incubators on a 12:12 light:dark cycle at 27 °C, and fed two 4th instar *Culex* larvae daily. The predator groups included *Pachydiplax* sp. nymphs (Odonata: Libellulidae), *Ischnura* sp. nymphs (Odonata: Coenagrionidae), and *Laccophilus* sp. larvae and adults

(Coleoptera: Dytiscidae), which were identified using taxonomic keys by Merritt et al. (2008), Epler (1996), and Wright and Peterson (1944).

Fig. 2.2 Outdoor treatment tubs used to expose predators to different levels of larvicide concentrations. One tub for each concentration (high, medium, low, and control). The plastic tent was used in case of rain, to keep water level constant for 24 hrs.

Predators were housed in plastic tubs (91.5 x 61.0 x 20.0 cm) and subjected to one of four levels of larvicide for 24 hrs: 0% (control, no chemicals), 10% of the recommended application concentration (low), the recommended concentration (medium), and double the recommended concentration (high) (Fig. 2.2). I chose to examine predators under the above larvicidal dosages to get a better idea of how these organisms react to different real-world scenarios. For example: the initial concentration of a chemical immediately after larvicide application (recommended) versus a low concentration of a chemical after it has had time to degrade. Double dosages were included to determine if there are any potential negative effects of these chemicals if none were seen in low and medium concentrations. Recommended concentrations for larvicidal chemicals are given on the product label as a set amount added per estimated surface area (Agnique Monomolecular

Surface Film®) or total volume of water (Altosid® Insect Growth Regulator). For SFs I used volume based off recommended concentrations and surface area of my treatment tubs: 10% = 0.025 mL, recommended = 0.25 mL, and double = 0.5 mL. For solid Altosid® IGR briquettes I based weights off the average weight of five briquettes (6.474 g) and recommended usage by volume of water being treated in my tubs: 10% = 0.0842 g, recommended = 0.8416 g, and double = 1.6832 g. There were four replicates for every predator and treatment combination.

Fig. 2.3 Predators in mesh pouches fastened to sides of tubs using thumb tacks.

Treatments (chemical + concentration) were conducted outdoors at the USM LTEC. Tubs were filled with 37.9 L of well water, and chemicals were added and homogenized

via stirring (Fig. 2.2). To prevent escape and any interaction among individuals during treatment, each insect was placed in a 14 x 6 cm mesh pouch made of Phifer© no-see-um fiberglass screening. Mesh pouches were fastened to the sides of the treatment tubs using thumbtacks and suspended halfway into the treatment water to provide access to the surface for respiration (necessary for *Laccophilus* adults) (Fig. 2.3). Individuals were subjected to chemical solutions for 24 hrs in the absence of food. Then, predators were removed from their pouches, rinsed, and placed into fresh water, and feeding behavior plus overall activity were observed on the same day post-treatment.

Fig. 2.4 Arrangement of viewing chambers in behavioral experiment.

2.2.2 Laboratory Observations

Clear acrylic containers (18 x 5 x 10 cm) were filled with 500 mL of reverse osmosis (RO) water. Two stalks of a common local aquatic plant (*Ludwigia palustris*) found in

roadside ditches and wetlands were added to each observation container (see Pitcher and Yee, 2014). Plant stalks were triple rinsed with tap water to remove debris and potential live invertebrates. Plant stalks, long enough to reach the bottom of the container, were suspended from a rectangular piece of cardboard fully covering each containers' opening (Fig. 2.4). A single predator was placed in each container, allowed to acclimate for 15 min, and then live prey (10, 4th instar *Cx. quinquefasciatus* larvae) were introduced.

Behavior and predation observations were recorded over 30 mins starting after the addition of prey. Observations, belonging to four categories, were recorded once per minute for every container during this time by a single observer (J. Nelsen): 1) Activity (swimming, walking, resting), 2) the surface they were touching (plant, wall, floor, or open space), 3) the predator's depth in the water column (at the surface, top ⅓, middle ⅓, or bottom ⅓), and 4) predatory activity (striking at prey, eating prey, or neither of these). Observations were recorded in a darkened room with a black curtain background and single light source positioned to illuminate all containers as evenly as possible (Fig. 2.4). Eight specimens were observed at a time, and cardboard pieces were placed in between observation chambers to eliminate any potential visual stimulus from neighboring chambers. Number of prey eaten was also recorded at the end of the observation period. Predators in all treatments were of same size/instar within orders to control for different feeding behaviors and energy requirements (Merritt et al. 2008). All four replicates of each predator and treatment combination were observed on the same day (e.g., reps 1-4 of damselflies in IGRs). As only eight containers were able to be observed at one time and there were a total of 16 individuals being observed on a given day, I observed replicates 1-2 of each concentration first, and placed replicates 3-4 in an incubator to

account for any behavioral differences that might be caused by time spent sitting in fresh water.

Behavioral effects of larvicides were determined based on the proportion of times they were observed performing each action over the 30 min period. After observational trials, the same individuals were reared over a week-long period to assess post-exposure survival. Individuals were placed in cups containing RO water and a wooden tongue depressor to provide structure, and stored in incubators set at 27 ºC, 12:12 light:dark cycle. Individuals were fed two mosquito larvae once per day.

2.2.3 Behavioral Analysis

To meet assumptions of normality, proportions of the behaviors within each category (activity, surface, depth, predation) were arc-sine square root transformed. A Principal Components Analysis (PCA) was then conducted on this transformed data, to reduce the number of correlated behaviors. A PCA was generated from each predator group, (i.e., damselfly, dragonfly, *Laccophilus* adults, and *Laccophilus* larvae), to account for natural behavioral differences among predators (Merritt et.al., 2008). Principal components (PCs) with eigenvalues ≥ 1 were retained for further analysis. Rotated factor loading scores (the degree to which each behavior is associated with a principal component axis) were analyzed in a two-factor factorial multivariate analysis of variance (MANOVA) with type (IGR, SF) and concentration (high, medium, low, and control) as factors. The results of the MANOVA were interpreted by examining Standard Canonical Coefficients (SCCs) generated by SAS, which help identify the PCs that are most responsible for any multivariate effects. Behaviors were considered important for a specific PC if loadings were $\geq \pm 40$ (Yee, 2010). To determine which PCs were being influenced by levels

within my independent variables (e.g., concentration = high, medium, low, or control), a Tukey's post-hoc analysis was conducted after each MANOVA. Effects of treatments on specific behaviors were then determined based on these PC loading scores. All analyses were performed in SAS (SAS Institute, Inc., 2004).

2.2.4 Survival and Predation Analysis

Assumptions of normal distribution were not met for one-week survival datasets in damselflies, *Laccophilus* adults, and *Laccophilus* larvae after conducting a Shapiro-Wilk's goodness of fit test. However, variance assumptions (homoscedasticity) were met for damselfly and beetle adult and larvae survival data by plotting residual versus predicted values and looking for patterns. As analysis of variance (ANOVA) is robust against departures from normality, I did not transform the data and instead conducted a two-factor factorial ANOVA to compare survival within predator groups (Blanca et al., 2017). Dragonfly nymph survival data was not analyzed because all individuals survived one week. Larval mosquito consumption data did not meet assumptions of normality, but variances were equal. Thus, a two-factor factorial ANOVA was conducted with larvicide type and concentration as factors and was used to compare prey consumption separately within each of the four predator groups.

2.3 Results

Table 2.1 Damselfly Behavior. Results of two-factor factorial MANOVA comparing PCA axes correlated with damselfly nymph swimming and hunting behavior among larvicide type, concentration, and interaction among concentration and type. Standardized Canonical Coefficients (SCCs) show amount of contribution each dependent variable in the MANOVA (when significant).

Source	df	Error df	Pillai's Trace	P-value	Standardized Canonical Coefficients			
					PC1	PC2	PC3	PC4
Concentration	12	69	0.578	0.200	- 0.453	0.943	- 0.064	0.592
Type	4	21	0.564	0.594	- 0.213	0.831	0.106	- 0.536
Concentration x Type	12	69	0.498	0.340	0.399	0.279	0.646	0.858

Table 2.2 *Laccophilus* Adult Behavior. Two-factor factorial MANOVA comparing first four PCs of *Laccophilus* adult swimming and hunting behavior among larvicide type, concentration, and interaction among concentration and type. Standardized Canonical Coefficients (SCCs) show amount of contribution of each dependent variable in the MANOVA (when significant).

Source	df	Error df	Pillai's Trace	P-value	Standardized Canonical Coefficients			
					PC1	PC2	PC3	PC4
Concentration	12	48	0.511	0.626	0.939	0.446	-0.193	0.623
Type	4	14	0.410	0.095	0.504	0.239	0.998	-0.505
Concentration x Type	4	14	0.349	0.169	0.949	- 0.425	0.558	-0.107

Table 2.3 Dragonfly Behavior. Two-factor factorial MANOVA comparing first four PCs of dragonfly nymph swimming and hunting behavior among larvicide type, concentration, and interaction among concentration and type. Standardized Canonical Coefficients (SCCs) show amount of contribution of each dependent variable in the MANOVA (when significant).

Source	df	Error df	Pillai's Trace	P-value	Standardized Canonical Coefficients			
					PC1	PC2	PC3	PC4
Concentration	12	69	0.671	0.095	0.453	-0.553	1.146	-0.042
Type	4	21	0.564	**0.001***	**1.064**	-0.801	0.687	-0.823
Concentration x Type	12	69	0.751	**0.046***	**-1.186**	1.068	-0.366	0.482

Table 2.4 *Laccophilus* Larvae Behavior. Two-factor factorial MANOVA comparing first four PCs of *Laccophilus* larvae swimming and hunting behavior among larvicide type, concentration, and interaction among concentration and type. Standardized Canonical Coefficients (SCCs) show amount of contribution of each dependent variable in the MANOVA (when significant).

Source	df	Error df	Pillai's Trace	P-value	Standardized Canonical Coefficients			
					PC1	PC2	PC3	PC4
Concentration	12	69	0.426	0.5012	1.343	0.009	0.249	0.951
Type	4	21	0.680	**<.0001**	**1.307**	-0.145	0.239	**1.020**
Concentration x Type	12	69	0.461	0.4191	-0.827	0.636	0.464	-0.687

2.3.1 Behavioral Analysis

Based on the PCAs, the first four PCs explained 78.48% of the variation in dragonfly behaviors, 73.59% of the variation in damselfly behaviors, 79.51% of the variation in *Laccophilus* larvae behaviors, and 77.77% of the variation in *Laccophilus* adult behaviors. For damselflies and *Laccophilus* adults, there were no significant effects of concentration, larvicide type, or the interaction of concentration and larvicide type on behavior (Tables 2.1 and 2.2, respectively). There was a significant effect of larvicide type and the interaction of type and concentration on dragonfly behavior (Table 2.3). There was also a significant effect of larvicide type on *Laccophilus* larvae behavior (Table 2.4).

Table 2.5 PCA Rotated Factor Pattern of Dragonfly Behaviors. Rotated factor pattern of first four Principal Components (PCs) generated by PCA on square root proportions calculated from dragonfly larvae behavior data. Absolute values deemed important (≥ 40) are in bold.

Category	Behavior	PC1	PC2	PC3	PC4
Activity	Rest	-26	-57	13	**-71**
	Walk	18	15	-21	**83**
	Swim	15	**95**	-7	12
Contact	Plant	**-89**	-32	-9	4
	Wall	6	**40**	**-46**	1
	Floor	**90**	10	16	-2
	Space	15	**95**	-9	10
Depth	Surface	0	0	0	0
	Top	-14	**44**	-11	**48**
	Mid	**-81**	-10	27	-19
	Bottom	**90**	-8	-19	-5
Predation	Strike	28	19	-38	**-62**
	Eat	-2	2	**-89**	20
	Neither	-8	-9	**96**	4

Table 2.6 PCA Rotated Factor Pattern of *Laccophilus* Larvae Behaviors. Rotated factor pattern of first four Principal Components (PCs) generated by PCA on square root proportions calculated from *Laccophilus* larvae behavior data. Absolute values deemed important (≥ 40) are in bold.

Category	Behavior	PC1	PC2	PC3	PC4
Activity	Rest	**-85**	24	-12	-15
	Walk	**78**	-28	11	3
	Swim	**61**	1	6	16
Contact	Plant	24	-3	**87**	32
	Wall	**40**	-2	-25	**67**
	Floor	1	**-92**	-34	-9
	Space	-16	**93**	-25	-13
Depth	Surface	-14	**94**	-21	-11
	Top	25	7	**80**	-9
	Mid	3	-2	29	**89**
	Bottom	9	**-93**	-30	-10
Predation	Strike	**62**	-13	**-42**	6
	Eat	**76**	5	**43**	4
	Neither	**-79**	-3	-39	-4

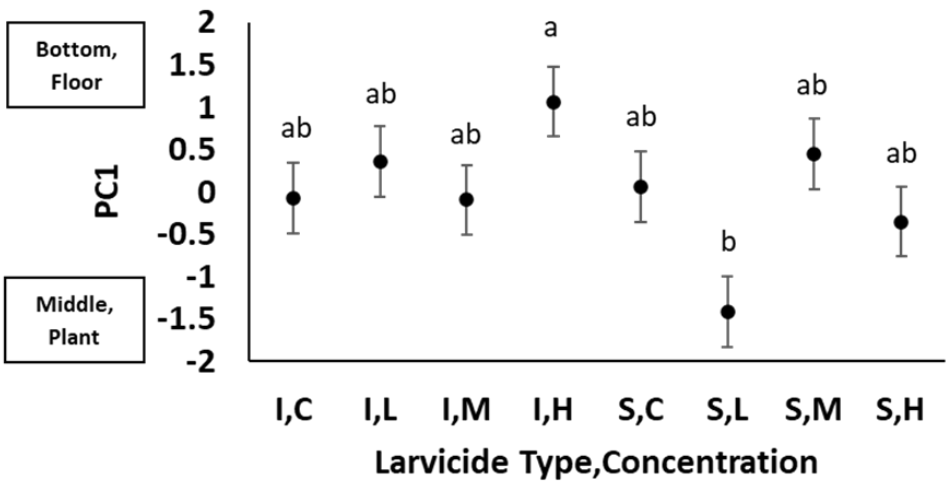

Fig. 2.5 Location preference (PC1) of dragonfly larvae among surface film (S) and growth regulator (I) treatments, and their concentrations (C = Control, H = High, M = Medium, and L = Low). Means (± 1 SE) that do not share letters are significantly different based on Tukey's post hoc adjustment. Behaviors strongly associated with PC1 are to the left of the Y-axis.

For dragonfly nymphs, PC1 separated individuals that occupied the bottom 1/3 of the water column and were in contact with the container floor (positive values) from individuals that spent more time on plants in the middle of the water column (negative values). For PC2, individuals were separated by swimming, being located on the wall, and in the top 1/3 of the water column (all positive values). Behaviors most strongly associated with PC3 were: neither striking at or eating prey (positive), and being located on the wall and eating prey (negative values). Principal Component 4 was separated by walking and being in the top 1/3 of the water column (positive values) and resting and striking at prey (negative values) (Table 2.5). There was no significant effect of concentration only on dragonfly behavior, but type and the concentration by type

interaction were significant (Table 2.3). Standardized Canonical Coefficients showed that PC1 was most important (Table 2.3). After conducting a Tukey's post-hoc adjustment, mean values for PC1 for larvicide type were significantly higher for IGRs (0.208) than mean PC1 values for SFs (-0.208). This means that dragonflies subjected to all concentrations of IGRs spent more time on the floor in the bottom 1/3 of the water column, and dragonflies subject to all concentrations of SFs spent more time on plants in the middle 1/3 (Fig. 2.5).

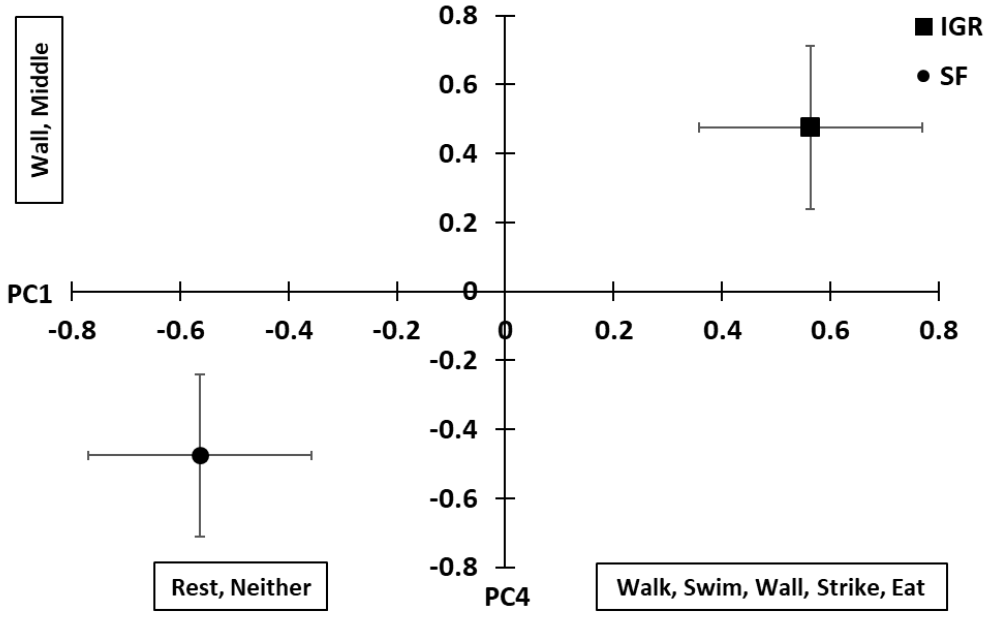

Fig. 2.6 Behavioral differences (PC1 = horizontal axis, PC4 = vertical) for *Laccophilus* larvae among combined concentrations of growth regulator (IGR) and surface film (SF) treatments. Mean of behaviors all replicates for PCs 1 and 4 (± 1 SE) by treatment type. Behaviors strongly associated with each PC are listed along the edges of the figure (e.g., "Wall" and "Middle" are negatively associated with PC1).

For *Laccophilus* larvae, PC1 was separated by individuals that spent more time walking, swimming, striking at prey, eating prey, and being located on the wall of the container (positive values), versus individuals that spent more time resting and neither striking nor eating prey (negative values). For PC2, behaviors were separated by not being attached to any substrate (space) and being at the surface of the water (positive values), versus being on the floor in the bottom 1/3 of the water column. Behaviors strongly associated with PC3 were eating prey, being located at the top 1/3 of the water column, attached to plants (positive values), and striking at prey (negative value). Principal Component 4 distinguished individuals that spent more time on the wall of the container, in the middle of the water column (positive values) vs. not being at these locations (Table 2.6). There was only a significant effect of larvicide type (IGRs vs SFs) on *Laccophilus* larvae behavior (Table 2.4). Standardized Canonical Coefficients showed that PCs 1 and 4 were most important (Table 2.4). After conducting Tukey's post-hoc adjustment, the PC1 value for IGRs (mean = 0.5629) was significantly greater than SFs (mean = -0.5629). For PC4, IGR values (mean = 0.4755) were also significantly higher than SFs (mean = -0.4755). Comparing these mean values to their associated factor loading scores: *Laccophilus* larvae in IGRs spent more time walking, swimming, striking at prey, eating prey, being located on the wall of the observation chamber, and in the middle 1/3 of the water column, and *Laccophilus* larvae in SF treatments spent more time resting and neither striking at or eating prey (Fig. 2.6).

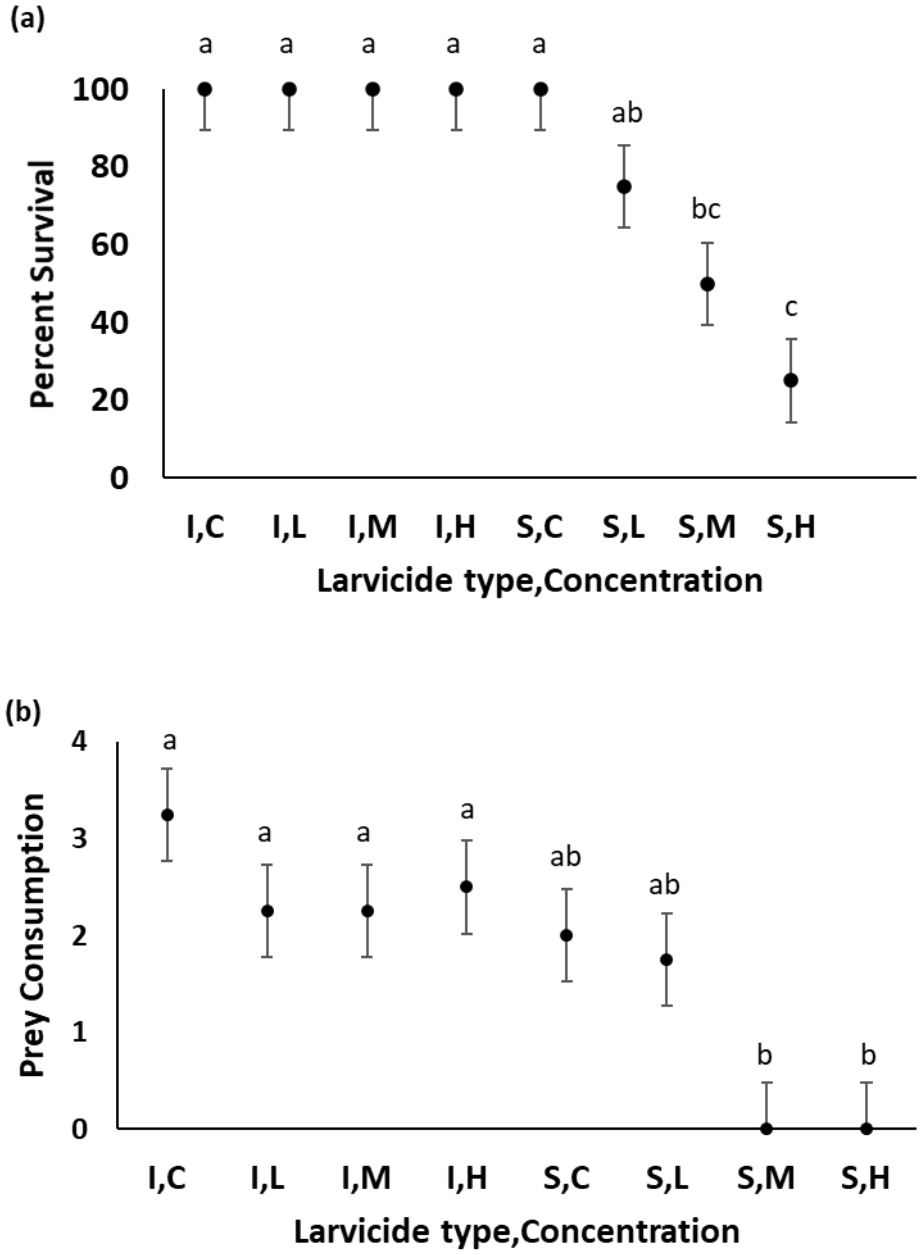

Fig. 2.7 Effects of larvicide type (surface film = S and growth regulator = I) and concentration (Control = C, Low = L, Medium = M, and High = H) on *Laccophilus* adult survival (a) and number of mosquito larvae eaten during 30 minute observation trials (b) (means ± 1 SE). For survival data, all *Laccophilus* adults that were dead had died on the day of treatment. The amount of prey consumed for dead adults was recorded as zero.

Treatments that do not share letters are significantly different based on Tukey's post-hoc adjustment.

2.3.2 Survival and Predation

All dragonfly larvae survived regardless of larvicide type and concentration (no analysis performed). There were no significant effects of larvicide type, concentration, or interaction of type and concentration on the survival of damselflies ($F_{7,31} = 0.868$, $p = 0.545$) or *Laccophilus* larvae ($F_{7,31} = 1.956$, $p = 0.104$). *Laccophilus* adults showed significant differences in survival ($F_{7,31} = 26.143$, $p = <.0001$). Specifically, adults in all IGR concentrations and SF low and control concentrations had significantly higher survival than *Laccophilus* adults in medium and high concentrations of SFs (Fig. 2.7a). Prey consumption with larvicide type and concentration as factors was analyzed within predator groups. No significant effects on prey consumption were found for damselflies: $F_{7,31} = 1.738$, $p = 0.148$, dragonflies: $F_{7,31} = 0.670$, $p = 0.695$, or *Laccophilus* larvae: $F_{7,31} = 1.635$, $p = 0.174$. As *Laccophilus* adults died in SF treatments, I initially analyzed prey consumption data by removing the dead adults and found no significant differences: $F_{7,22} = 0.7089$, $p = 0.6249$. I conducted this same analysis, but instead adding zeroes for the number of mosquitoes consumed by the dead adults, which showed significant differences: $F_{7,31} = 5.922$, $p = 0.0004$. After conducting a Tukey's post-hoc adjustment, *Laccophilus* adults in all IGR treatments (H, M, L, C) ate more mosquito larvae than adults in medium and high SF treatments, with control and low SF adults as an intermediate between all IGRs and M and H SFs (Fig. 2.7b).

2.4 Discussion

I examined behaviors of four different predators after being exposed to different concentrations of two common mosquito larvicides, surface films (SF) and insect growth regulators (IGR). I hypothesized that predator behavior will vary by larvicide type and concentration, and that there would also be differences in behavior when comparing larvicide-treated individuals to control individuals. I found that in the presence of chemicals, dragonflies occupied different surfaces and depths when comparing behaviors of all combined concentration replicates of SFs (plant, middle 1/3) to all IGR replicates (floor, bottom 1/3). These results suggest that something about these chemicals might be causing dragonfly larvae to act differently. There were also significant effects of the interaction of larvicide type and concentration on dragonfly behavior (Tables 2.5 and 2.6). However, these results were deemed irrelevant as the only significant differences were seen across larvicide types (i.e., high IGR vs. Low SF), not among different concentrations of the same type. The purpose of including the interaction effect was to help determine if varying concentrations of the same larvicide changed how an insect behaves, and differences among concentrations of different larvicides do not help answer that question.

Laccophilus larvae exhibited different behaviors when comparing all IGR concentration treatments to all SF concentration treatments. This included controls, and after removing control treatments and conducting the PCA again, the same results were generated. Over all concentrations combined, *Laccophilus* larvae were resting more and eating less in IGRs, and swimming, walking, striking, and spending more time eating in SF treatments. They were also more often on the wall in the middle 1/3 of the water

column in all IGR treatments when compared to all SF treatments. This may suggest that IGR treatments caused *Laccophilus* larvae to be more lethargic than SF treatments.

There were no significant behavioral effects of any larvicide type and concentration on damselfly larvae or *Laccophilus* adults. However, there was a lethal effect that SFs had on *Laccophilus* adults at recommended and higher concentrations. *Laccophilus* larvae showed significant differences in behavior when comparing larvicide type only. As suggested by my experiments, IGRs and SFs may change the swimming behavior in predatory insects like aquatic beetle and dragonfly larvae. Why were there no behavioral effects seen in damselfly larvae and *Laccophilus* adults? One explanation is that damselfly and dragonfly nymphs both have biological gills (Merritt et al. 2008) and thus do not need to interact with the surface as often as beetles. However, this does not explain why behavioral differences were seen between larvicide types in dragonflies. Although dytiscid larvae have gills, they can also siphon oxygen from the atmosphere (Kehl, 2014), which is likely why immediate mortality was only seen in adult beetles. If SFs likely remove the option of siphoning in dytiscid larvae, then they are forced to use only one method of obtaining oxygen, which could have been a reason for differences seen in behavior among larvicide types. All *Laccophilus* adults exposed to high and medium concentrations of SFs died (including one in low concentration) before I could analyze their behavior. The lack of a full data set prevented me from comparing behavioral effects between larvicide types in *Laccophilus* adults (there were four alive in controls and three alive in low concentrations). Given that SFs were effective at killing adult beetles does make examinations of their behavioral differences somewhat moot.

For insect survival after exposure to larvicides, I hypothesized that survival would differ based on the larvicide type and concentration. Miura and Takahashi (1973) observed an LC50 of methoprene (IGR) at 2.0 ppm in *Laccophilus* adults. However, this study used a sustained release of liquid methoprene in a laboratory setting for 48-72 hrs. For my experiments, I exposed all predators to crushed solid methoprene briquettes, mixed in large plastic tubs for 24 hrs. Therefore, even though my concentrations were higher than the LC50 described in Miura and Takahashi (Low = 2.23 ppm, Medium = 22.23 ppm, High = 44.47 ppm) the *Laccophilus* adults in my study likely survived due to these differences. In addition to this Miura and Takahashi (1984), also found no significant changes in *Laccophilus* mortality after collecting individuals from sites that were recently treated with IGRs. In medium and high concentrations, SFs prevented these beetles from utilizing the water's surface tension and accessing the atmosphere, which likely starved them of oxygen (Merritt et al, 2008). In contrast, survival was higher in controls and low concentration because the oil layer was likely thin enough for the beetles to still gain access to atmospheric oxygen, or there was not enough oil to cover the entire surface area in the tub. Although this showed that SFs are lethal to *Laccophilus* adults in an artificial setting, I was not able to determine how this larvicide affected beetle adults in the wild based on surveys from Chapter I of this thesis.

I recorded the number of 4th instar *Culex* mosquito larvae eaten (out of 10) by each predator after the behavioral trials. For this, I hypothesized that there would be differences in mosquito consumption within predator and larvicide type groups based on the concentration they were exposed to. I found no significant differences in prey consumption across 3 of the predator groups (damselflies, dragonflies, and *Laccophilus*

larvae). No *Laccophilus* adults survived in medium and high treatments of SFs, thus, prey consumption was marked as "zero" for the analysis. For *Laccophilus* adults, there were significant differences in mosquito mortality between high and medium SF treatments and every other treatment level and concentration combination. These results suggest that in this laboratory setting, unless directly lethal, larvicides did not affect prey consumption in these predatory groups.

This is the first known study to directly observe the behavior of dragonflies, damselflies, and aquatic beetle juveniles and adults after being subjected to IGRs and SFs. This study showed that recommended amounts of SFs are directly lethal to *Laccophilus* sp. adults, likely in the same manner they are intended for killing mosquito larvae (blocking atmospheric access). This genus of diving beetles is relatively small (my specimens ~4.5 mm length), whereas adults in the entire family of Dytiscidae range 1-45 mm in length (Yee, 2014). Surface films may have different effects depending on the size of the animal. For instance, a dytiscid from a larger genus may be able to break through the oil barrier and siphon air. In addition to predaceous diving beetles, there are other aquatic insects that siphon air, which include predatory Hemiptera like corixids, notonectids, *Toxorhynchites* spp. larvae, belostomatids, and other beetles whose larvae are predatory (e.g., Hydrophilidae), all of which have been shown to prey on mosquito larvae (Shaalan and Canyon, 2009). Experiments examining the direct lethality and sublethal behavioral effects should be conducted on individuals belonging to aquatic insects like these.

Future work could examine non-lethal effects of IGRs non-target organisms. Suffocants like SFs likely affect a much wider variety of organisms, but more research

should be done to determine specifically what animals, and to what degree, SFs harm mosquito predators. Surface films can still be necessary when many mosquitoes need to be killed in a short amount of time. For example, during public health emergencies, after natural disasters, or in areas where there are high rates of mosquito-vectored pathogens. There are many other invertebrates that rely on surface tension to move around and gather food (e.g., water striders, semi-aquatic spiders, whirligig beetles). As animals like these spend their entire lives on or near the water's surface, other semi-aquatic insects rely on it for completing their life cycle. For oviposition, aquatic flies need to be able to land on the water to lay their eggs, and odonates need to be able to dip their abdomens under water. Odonate nymphs, which do not siphon atmospheric oxygen, still need to break through the surface when emerging from their final molt into adulthood. Do SFs affect how these invertebrates perform these tasks? This is something that should be addressed to gain a better understanding of the effects that SFs have on aquatic invertebrate communities.

If sublethal effects on non-targets do occur in the wild, larvicides may significantly alter food webs because any impairment or developmental effects on an individual that reduces its ability to hunt and acquire food may also reduce that individual's effectiveness to control pests in that system (Desneux et al., 2007; Douglas et al., 2015). The preservation of known mosquito-eating non-target organisms is important for both maintaining balance of the trophic web of the habitats they live in and the regulation of pathogen vectoring mosquito species (Connelly and Carlson, 2009; Culler and Lamp, 2009; Kumar and Jiang-Shiou, 2006; Merritt et al., 2008).

CHAPTER III: LARVICIDES AND PREDATORY RELEASE

3.1 Introduction

Aquatic predators including predaceous diving beetles (i.e., Coleoptera: Dytiscidae), predatory mosquito larvae (*Toxorhynchites* spp.) (Diptera: Culicidae), and Odonates (Order: Odonata) play an important role in naturally regulating mosquito populations (Connelly and Carlson, 2009; Kumar and Jiang-Shiou, 2006; Shaalan and Canyon, 2009). Copepods (Arthropoda: Cyclopoida) are also known predators of first instar mosquito larvae (Brown et al., 1991). Larval development time for mosquitoes and their predators are heavily dependent on temperature, food quality, and food availability (Clements, 1999). Development time of the genera *Aedes* and *Culex* generally range from seven to 15 days in typical summer and breeding season temperatures (20 to 23 ºC) (Buth et al., 1990; Clements, 1999; Rueda et al., 1990; Tun-Lin et al., 2000). Development time of predaceous diving beetle larvae (Coleoptera: Dytiscidae) varies depending on the species, as short as two weeks to multiple months (Arnold et al., 1998; Miller and Bergsten, 2016). Species of dragonflies and damselflies (Order: Odonata) vary in juvenile development time as well, ranging from weeks, to months, to years (Evans, 2007; Plaistow and Siva-Jothy, 1999; Suhling et al., 2004). Killing or harming predatory taxa via larvicide application could relieve predation pressure on mosquito populations and create higher larval abundances in previously treated habitats if sites are not regularly treated, as the presence of a predator lowers the maximum amount of larval mosquitoes that can live in a habitat (Chase and Shulman, 2009). Pesticides are designed to degrade over time, and when chemical concentrations dissipate (which is accelerated by factors like rain, soil uptake, sunlight exposure, microorganisms, and pollution), organisms that

are normally affected are able to recolonize (Randall, 2006). Conversely, evaporation of treated standing water during a dry period might increase the concentration of a pesticide. Lower predation pressures may reduce mosquito mortality and increase overall fitness, by both lowering direct predation and giving prey more time to consume resources instead of avoiding predators (Awasthi et al., 2015; Kesavaraju et al., 2007; Werner, 1991).

This chapter aims to test the hypothesis that there will be a difference in the abundance of emerging mosquito adults among mesocosms containing predators exposed to Insect Growth Regulator (IGR) and Surface Film (SF) larvicides, mesocosms with unexposed predators, and negative control mesocosms not containing predators. I hypothesized this because, as stated in my previous chapters, past research has shown that pesticides can reduce the hunting ability of non-target predators (Ahmad et al., 2003; Claver et al., 2003; Martinou et al., 2014). To address this hypothesis, I constructed artificial habitats that simulated shallow ephemeral pools and assessed the ability of non-target predators to suppress larval mosquito populations after exposure to larvicides. From this hypothesis and past research listed above, I predicted that mosquito survival would be highest in mesocosms containing no predators (negative controls), lowest in mesocosms containing predators not exposed to larvicides (IGR and SF controls), and range in increasing level of larvicide concentration (e.g., lower mosquito survival in low concentrations of SFs and IGRs, and higher mosquito survival in presence of predators that received higher dosages of SFs and IGRs). In terms of differences in mosquito emergence between the two larvicide types, I predicted that mesocosms containing SF-treated predators would produce more mosquitoes, because in previous behavioral trials (Chapter II of this thesis), *Laccophilus* adults died after 24 h in medium and high

concentrations of SFs (while every other predator group in every other treatment, including *Laccophilus* adults in IGRs, remained alive). Thus, higher mortality of predators would likely allow more mosquito larvae to pupate and emerge as adults.

In addition to these outdoor mesocosm experiments, I examined one-on-one intraguild predation (IGP) among my four predator groups in a laboratory setting, in the presence and absence of mosquito prey. Intraguild predation happens when one organism kills and eats a potential resource competitor, and can occur across taxa, even among members of the same species (Polis et al. 1989). In some systems, IGP can be a large portion of an organism's diet and can occur more frequently in the absence of common prey items (Polis et al. 1989). Cannibalism and IGP among predaceous aquatic insects are common in ephemeral wetlands and is likely an important factor that regulates their population size and density (Batzer and Wissinger, 1996). Thus, I hypothesized that IGP was more likely to occur in the absence of mosquito prey. To test this hypothesis, I placed two individuals (combinations of one of the four predator groups: *Laccophilus* adults, *Laccophilus* larvae, damselflies, and dragonflies) in small plastic containers. I recorded predator mortality, as well as mosquito larvae mortality in replicates that contained predators and mosquitoes. For these tests I predicted that if IGP occurred, the smaller predator would always be the prey (e.g., damselflies would eat *Laccophilus* larvae, dragonflies would eat damselflies). I also predicted that even though *Laccophilus* adults are larger than their larvae, they likely would not consume each other. Even though cannibalism has been shown to occur among adult and larval beetles of the same species (Deding,1988; Hicks,1994), I predicted it would likely not happen in these laboratory

experiments because the amount of time I decided to starve all predators for (24 h) might not be long enough to induce this behavior.

Lastly, I made sure that mosquito larvae mortality was not being influenced by the contamination of larvicides during the movement of predators from their treatment tubs to the experimental mesocosms. To test this, I collected water from each mesocosm tub after the introduction of all predators, and reared extra mosquito larvae (from the same source egg rafts as the mesocosms) in these separate samples. For these tests, I hypothesized that there would be no differences in the number of emerged mosquitoes among all water samples. I predicted that there would be no differences because, if I had properly rinsed every predator and took necessary precautions to prevent larvicide contamination while transferring them, then there would be no chemicals in these water samples to affect mosquito emergence.

The information gained from these experiments will be useful for future research in mosquito biocontrol. The mesocosms used in this study were designed to simulate the depth and plant density of a roadside ditch, while still being able to collect all adult mosquitoes that emerge. Knowing how emergence rates differ among habitats, with the only variable being chemical exposure to the introduced predators, will provide a better understanding how larvicides affect the ability of these insects to naturally control mosquito populations.

It is also important to address the tradeoffs of using mesocosms to simulate a larger body of water. While this method of only exposing predators is not comparable to conditions seen in a real-world scenario (i.e., mosquitoes and their predators would be exposed, predator and prey densities by volume in mesocosms may not reflect their

natural densities), these experiments were designed to provide insight into the effects of these larvicides on the mosquito-controlling capabilities of predators. If mosquitoes had been exposed, then there would be no effective way to determine if their mortality as juveniles and emergence rates had been due to predation or chemical effect. The use of mesocosms in this study also helped control for outside factors that might influence larvicide efficacy (e.g., sunlight, water volume, microbes, pollution), predatory behavior and survival (e.g., insectivorous vertebrates), and mosquito survival (e.g., controphic invertebrates, detrital amount).

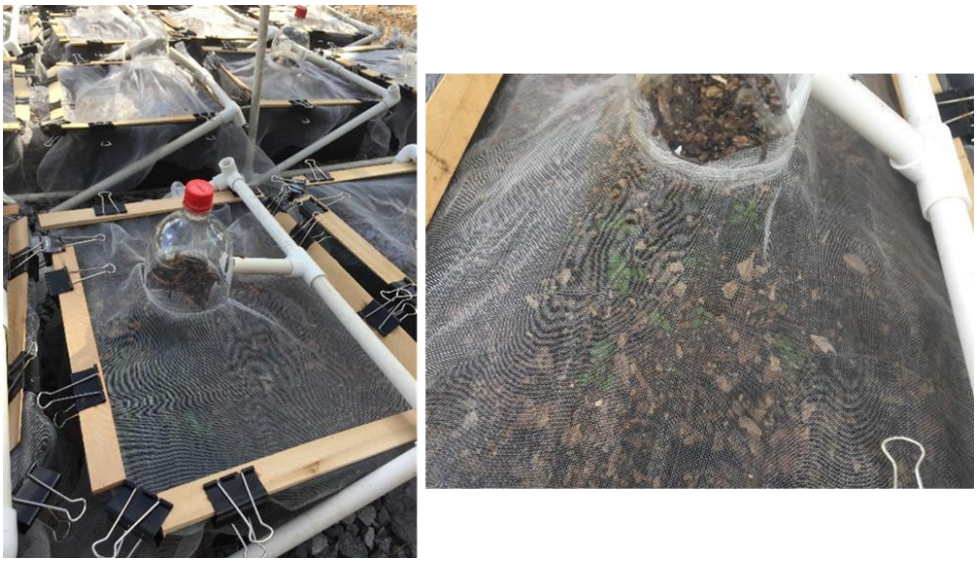

Fig. 3.1 Left: A group of mesocosms with bottle traps not yet attached. Right: A close-up of a mesocosm containing aquatic plants, leaf detritus, and aquatic insects.

3.2 Materials and Methods

3.2.1 Collection and Treatments

Mosquitoes of the genus *Culex,* which are generally associated with open water habitats like roadside ditches and wetlands (Molaei et al., 2007), were used as prey. *Culex*

egg rafts were collected in the wild from containers filled with a solution of fish meal and water as oviposition bait in plastic tubs. Rafts were collected at the University of Southern Mississippi Science Park (31.353155°N, -89.358994°W) and the LTEC. Experiment were also conducted outdoors at the LTEC. Artificial habitat mesocosms (50.8 x 38.1 x 17.8 cm plastic tubs) were filled with 24 L well water (15.2 cm deep) and 24 stems of *Ludwigia palustris* to simulate a shallow roadside ditch (plant density based on experiment in Bofill and Yee, 2019). Dried senescent leaves of three common trees collected around Lake Thoreau (9.95 g each: water oak (*Quercus nigra*), live oak (*Quercus virginiana*), and maple (*Acer* sp.) were added to each mesocosm as a food source for mosquito larvae. Leaf amount by volume was calculated based on experiments done by Muturi et al. (2012). Water (120 mL) from a pond at Petal River Park and 120 mL filtered cricket water (250 mL dried crickets soaking in a 5-gal bucket one day prior) were added to each mesocosm to stimulate biofilm growth on leaf detritus (Fig. 3.1).

Mosquito larvae were reared in incubators set at 27 ºC, 12:12 light:dark cycle and fed ground dog food based on the daily amount per larvae used in Gerberg et al. (1994). Detritus and nutrients were allowed to soak 72 hrs before the addition of mosquito larvae. One hundred and fifty *Culex* larvae (3^{rd} instars) were then added to mesocosms to serve as a food source for predators. Third instar mosquito larvae were used to avoid mortality that may be caused by factors other than predation (e.g., temperature shock when being added to mesocosm, physical trauma when being transported from the USM campus lab to the LTEC, static field from the plastic mesocosms). On the day that mosquitoes were added, I also began the predator larvicide treatments. Dog food was added to each

mesocosm for the next four days to ensure mosquito mortality was only due to predation, and not a lack of nutrients.

Fig. 3.2 Treatment tubs with plastic covering used to expose predators to varying concentrations of larvicides in mesocosm experiment.

Fig. 3.3 Mesh pouches used to contain predators during larvicide treatments prior to introduction into mesocosms. Pouches were suspended in water using paper clips attached to a long PVC pipe.

One week prior to the start of each mesocosm experiment, predators were collected from the same untreated sites mentioned in Chapter II and kept alive using the same methods prior to the day of treatment. Two individuals from each predator group

(dragonfly, damselfly, *Laccophilus* larvae, *Laccophilus* adult) were added into mesocosms simultaneously after being subjected to larvicides for 24 h using similar treatment methods as the previous behavior experiment. Treatment tubs were modified to hold more predator pouches at a time by placing PVC tubes across four tubs that were lined up horizontally (Fig. 3.2). Holes were drilled into these PVC tubes, and a paper clip was inserted with one end bent around the PVC, and the other bent into a hook-shape. On the hook-ends of these paper clips, another paper clip was attached, which were used to close and suspend mesh predator pouches halfway into the water (Fig. 3.3). Immediately after removing these insects from the treatment tubs, each individual was triple rinsed with on-site well water to reduce the potential of larvicide contamination in the experimental mesocosms. Four replicate mesocosms were used for each larvicide and concentration combination used in the previous toxicity experiments. In addition to control treatments (no chemicals with predators and mosquitoes) to determine mosquito survival in the presence of unaffected predators, I used negative controls (no chemicals with mosquitoes and no predators) to quantify natural larval mortality and emergence of adult mosquitoes. To avoid overfilling from rain, I drilled six small holes around the perimeter of each mesocosms' 24 L depth mark. Holes were small enough to prevent predators or adult mosquitoes from escape.

Fig. 3.4 Sticky card trap in bottle with adult mosquitoes attached.

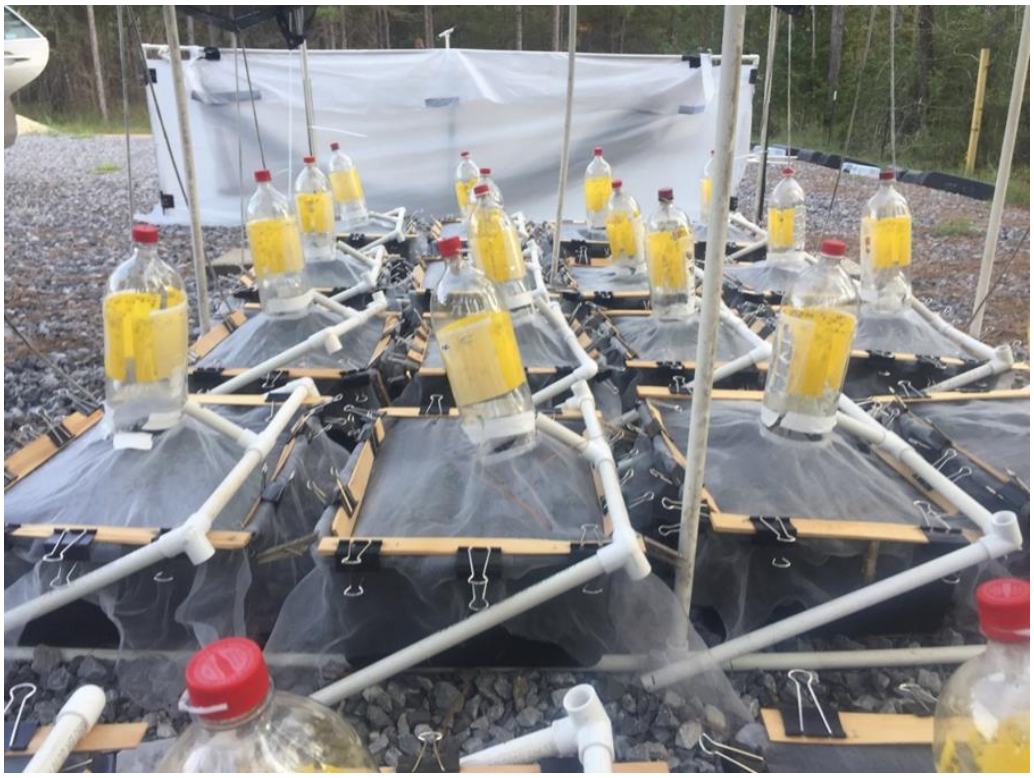

Fig. 3.5 Mesocosm replicates under black screen shelter. Bottle traps with yellow sticky cards attached.

3.2.2 Mosquito Emergence

Emerging mosquito adults were collected in traps that covered each mesocosm. Emergence traps were constructed using white garden mesh, and a clean plastic 2 L soda bottle attached to central hole cut in the mesh (Fig. 3.1). Plastic bottles had their bottoms cut off and the top 1/3 of the bottle attached to the mesocosm and netting, creating a funnel into the trap that is easier for an insect to enter than exit (Fig. 3.4). Bottle traps were pushed firmly down onto the mesocosm bottle, and gaps were sealed around the perimeter where the two bottles joined with labeling tape to prevent adult mosquitoes from escaping and outside insects from entering (Figs. 3.4 and 3.5). To further ensure adult mosquitoes did not escape, I cut yellow Garsum© sticky cards in half and placed

both halves on the inside of each bottle (Figs. 3.4 and 3.5). I also attached a piece of cotton soaked in 10% sucrose solution inside the caps of each bottle to attract adults upwards once they emerged. The plastic bottle/net combination was held above each mesocosm using PVC frames as support, allowing the netting to drape over the edge of the mesocosm and taper up towards the bottle (Fig. 3.1). Netting was fastened to the edge of each mesocosm using wood shims and metal binder clips to seal off gaps that were large enough for insects to enter or escape through. The experiment concluded after 15 d as most larvae would have pupated in that amount of time (maximum pupation occurs within 8-9 d for *Culex* larvae while being lab reared under ideal conditions, (Gerberg et al., 1994)). Sticky cards containing adult mosquitoes were removed from the bottle traps and adults on cards were counted in the laboratory under a dissecting microscope. A few adult mosquitoes died before flying into the bottle trap and degraded in the water in almost all replicates. I was able to find their individual thoraxes floating at the surface which were added to the total count. I had a total of 40 mesocosms (two chemicals, four replicates of each larvicide by concentration combination, and eight negative controls), but it was most feasible to run 20 at a time (Fig. 3.5). To avoid differences in mosquito emergence that might arise from seasonality (e.g., temperature, photoperiod), I ran the first two replicates of both larvicidal chemicals (SF, IGR) along with four negative controls at the same time. Survival of predators was recorded immediately after treatments, before they were placed into their respective mesocosms.

3.2.3 IGP Experiments

I used predators collected from the same sites in the mesocosm experiments. These predators included *Laccophilus* adults, *Laccophilus* larvae, dragonfly nymphs, and

damselfly nymphs. There were three treatment levels within each of the above pairings: predators only, predators with 10 mosquito larvae, and a negative control of 10 mosquito larvae only to quantify their natural mortality during trials. Mosquito larvae used were 4th instar lab reared *Culex* larvae that were collected from egg rafts around Hattiesburg, MS. To keep predators alive prior to the experiment, they were kept in 250 mL Tri-Pour cups (one individual per cup) containing 200 mL RO water and a piece of a wooden tongue depressor for perching, fed two 4th instar larvae daily. Predators were starved for 24 h prior to experiment start.

Experiments began when predators were simultaneously placed in 400 mL Tri-Pour cups containing 375 mL RO water and a piece of a tongue depressor to simulate structure. Mosquito larvae were introduced into these cups before predators were added. A square section of black mesh was used to cover each cup and prevent individuals from crawling out (mostly necessary for *Laccophilus* adults). There were five replicates of each of the three treatment levels (predators only, predators with 10 mosquitoes, and 10 mosquitoes only) which were placed on plastic trays and left in an incubator for 4.5 hr. Predator and mosquito mortality was then recorded. These experiments were conducted at the USM campus inside incubators on a 12:12 light:dark cycle at 27 °C.

3.2.4 Contamination Test

To ensure any changes in mosquito adult emergence from the mesocosm experiments were due to predation and not contamination of larvicides, I reared more mosquito larvae in water samples taken from each replicate. One day after introduction of predators, I collected 100 mL samples using Fisher Scientific© specimen cups from all 40 mesocosms. These samples were placed in an incubator and 10 4th instar *Culex* larvae

(extra larvae hatched from same egg rafts of larvae used in these mesocosms) were placed in each 100 mL cup. Larvae were fed daily dog food per larvae as specified in Gerberg et al. (1994). Observations ended when all pupae had eclosed, or when all larvae or pupae had died.

3.2.5 Mosquito Emergence Analysis

Mosquito emergence among mesocosm replicates of different treatment levels was compared using a one-factor ANOVA in order to include negative control mesocosms into the analysis, as negative controls were a treatment type that contained only one concentration level (larvicide-free controls). Thus, treatment levels consisted of fully crossed combinations of treatment type (IGR, SF) and concentration (H, M, L, C) plus the negative control mesocosms. All data analyzed in this chapter were first tested for assumptions of normality using Shapiro-Wilk's Goodness of Fit test, and assumptions of homoscedasticity by plotting residual against predicted values and looking for patterns. Emergence data met assumptions of homoscedasticity and normality.

3.2.6 Treatment Survival Analysis

Predator survival post-treatment was compared among replicates of larvicide type and concentration, within each of the four predator groups. All damselflies and dragonflies survived treatments; thus, they were not analyzed. Variances were equal for survival data for *Laccophilus* adults and larvae, but did not meet the assumptions of normal distribution. As ANOVAs are robust against departures from normality, I conducted a two-factor factorial ANOVA with larvicide type and concentration as factors comparing survival of *Laccophilus* adults and larvae, separately.

3.2.7 IGP Analysis

I analyzed data from the IGP experiments by first determining if predator mortality occurred in replicates of the predator pairing combinations. None of this data met assumptions of equal variances. Thus, in pairings where IGP was present, I conducted a non-parametric Wilcoxon/Kruskal-Wallis Rank Sums test to determine if treatment groups (i.e., predators only vs. predators with mosquitoes) were significantly different from each other in terms of predator mortality. I conducted These same analyses were applied to compare mosquito mortality in predator treatments to negative control cups containing 10 mosquitoes only.

3.2.8 Contamination Test Analysis

Adult emergence data from the residual larvicide contamination experiment did not meet assumptions of normality but did meet assumptions of homoscedasticity. Emergence was compared among larvicide type and concentration combinations and negative control treatments using a one-factor ANOVA, as ANOVAs are robust against departures from normality. The treatment levels were the same as the one-way analysis used in the main emergence experiment.

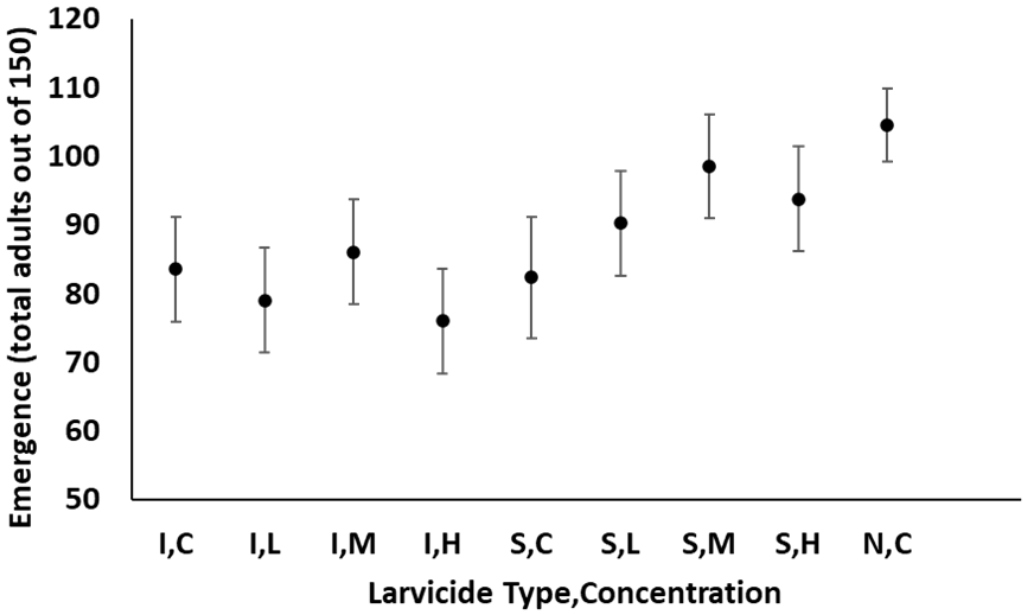

Fig. 3.6 ANOVA results comparing mean (± 1 SE) adult mosquito emergence from replicates of treatment type (SF = S, IGR = I, and negative control = N) and concentration (Control = C, Low = L, Medium = M, and High = H) combinations.

3.3 Results

3.3.1 Mosquito Emergence

There were no significant differences in mosquito emergence among mesocosms that contained predators treated with all concentrations of SFs and IGRs, as well as negative control mesocosms containing no predators after conducting a one-way ANOVA ($F_{8,30}$ = 1.997, p = 0.082) (Fig. 3.6).

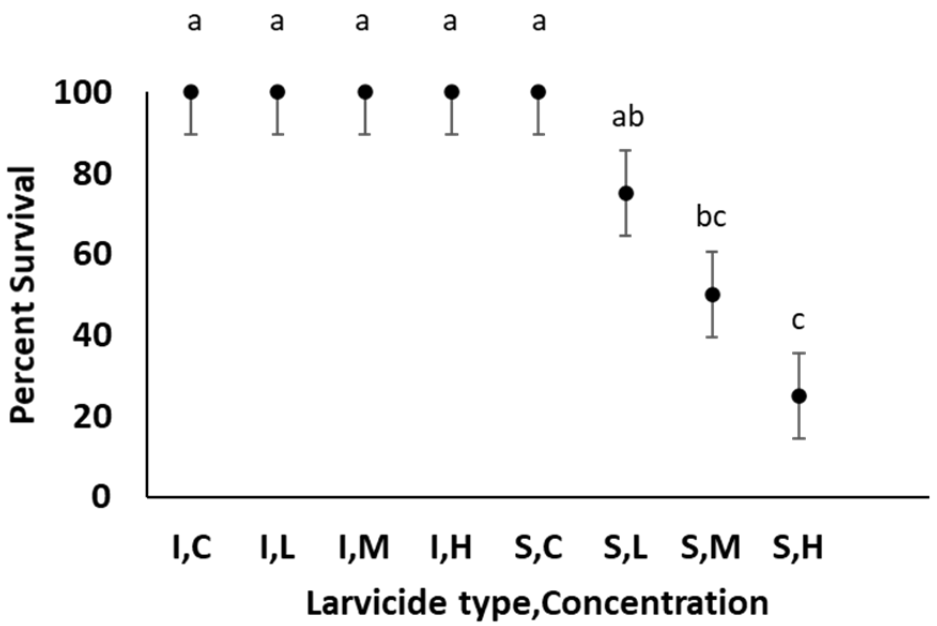

Fig. 3.7 Mean percent survival of *Laccophilus* adults (± 1 SE) among larvicide type (SF = S and IGR = I) and concentration (Control = C, Low = L, Medium = M, and High = H) combinations. Treatments that do not share letters are significantly different based on Tukey's post-hoc adjustment.

3.3.2 Predator Survival

All damselflies and dragonflies remained alive during the experimental trials. One *Laccophilus* larvae in high SF treatments died, and no significant differences were seen among all treatment combinations ($F_{7,63} = 1$, $p = 0.4411$). There were significant differences in survival among *Laccophilus* adults among treatments ($F_{7,63} = 7.6$, $p = <.0001$). Specifically, all IGR treatments and the control SF treatment had significantly higher survival of adult *Laccophilus* (100% alive) than medium (50%) and high (25%) concentrations of SF. Low SF survival (75%) was intermediate between medium SF and the above treatments that had 100% survival. Lastly, high SF treatments had lower

survival when compared to low SF, with medium SF as an intermediate between these two (Fig. 3.7).

3.3.3 IGP

Predation between predators only occurred within pairings of dragonfly nymphs and *Laccophilus* larvae, and damselfly nymphs and *Laccophilus* larvae. In all replicates, *Laccophilus* larvae were the prey. Also, *Laccophilus* larvae mortality only occurred in the absence of mosquito larvae (versus damselflies 3/5 replicates were eaten, versus dragonflies 4/5 replicates were eaten). Neither pairings were normally distributed, so I conducted Wilcoxon/Kruskal-Wallis Rank-Sum tests to determine differences between predator only replicates and predator with mosquito prey replicates. Chi-square approximations were used due to low replicate numbers. Predator only and predators with mosquito treatments were significantly different from each other in both pairings. Damselflies vs. *Laccophilus* larvae: $\chi^2 = 6.0000$, df = 1, Prob > $\chi^2 = 0.0495$. Dragonflies vs *Laccophilus* larvae: $\chi^2 = 3.8571$, df = 1, Prob > $\chi^2 = 0.0143$.

3.3.4 Contamination Test

There were no significant differences in adult mosquito emergence among larvae reared in water samples from replicates of mesocosm treatments (SF and IGR concentrations and negative controls) ($F_{8,31} = 0.621$, $p = 0.754$).

3.4 Discussion

I examined adult mosquito emergence from mesocosms containing predators that were exposed to different concentrations of SFs or IGRs, predators from control treatments, and negative controls with no predators. I hypothesized that mosquito emergence would vary based on the treatment (SFs, IGRs, and their respective

concentrations) of their predators, as well as presence and absence of predators (controls vs negative controls). One important caveat is that the combination of predators, mosquito larvae, and plants does not represent the full community of organisms found in roadside ditches. The natural sites that these simplified mesocosms were designed to replicate contain many other organisms that interact with the taxa used in these experiments, including: vertebrates (e.g., wading birds, reptiles, amphibians, occasionally fish), non-insect invertebrates (crustaceans, semi-aquatic spiders, worms, protists), and a wide variety of aquatic plants and algae. Results suggest that at this density and combination of predators used there is no significant effect of larvicide type and concentration on larval mosquito mortality. Because there were also no significant differences in mosquitoes collected among negative controls and controls containing predators, the predators themselves likely did not eat larvae at rates to affect the number of adults that emerged from these mesocosms, regardless of exposure to larvicides. However, emergence did approach significance ($p = 0.082$). Negative controls had the highest mean emergence overall of 104.5 adults captured. I predicted that lowest mosquito survival would be seen in controls that contained predators, however the lowest emergence came from IGR-H mesocosms at 76 adults, with IGR-C and SF-C near the middle of all treatments (83.5 and 82.3 adult mosquitoes, respectively). All SF chemical treatments had higher emergence than all IGR chemical treatments, which aligns with my predictions of higher mosquito emergence in the presence of SF-exposed predators (albeit still non-significant).

Before placing predators into mesocosms, I noted mortality post-treatment. Overall, six *Laccophilus* adults died in high SF, four died in medium SF, and one died in low SF.

One *Laccophilus* larvae died in high SF. There were three instances where a predator became stuck in a mesh pouch and died while I was trying to remove it: two damselflies in low SF and one *Laccophilus* larvae high IGR. There was one instance of a *Laccophilus* adult escaping from a mesh pouch during overnight treatment, which was from a low SF replicate. Also, in all four replicates there was a slow leak in the low SF treatment tub, which resulted in a lower water level when I returned the next morning to transfer individuals to their respective mesocosms. The water level was low enough to be noticeable, but the bottom ¼ of the pouches in this tub were still submerged.

There could be a few reasons for the lack of differences in mosquito emergence. One explanation is that predator and prey densities in these mesocosms were too low to allow predators to significantly reduce mosquito numbers, but this may not be the best explanation as my experimental densities could be considered higher or lower than in nature. The density and invertebrate community structure of an ephemeral pool depends on a variety of factors (e.g., days after site inundation, time of year, locality, pollution) (Walton, 1990; Williams, 1996). In field surveys from Chapter I, the number of insects among individual sites greatly varied from hundreds to less than 10 (note that Table 1.6 is the *total* amount of individuals collected from four replicates of each treatment). It could have also been due to the time of year that I ran this experiment. I collected my predators during mid-late fall in southern Mississippi, and the lower temperatures and shorter photoperiod might have influenced their behavior, appetite, or energy demands. Daily average temperature was dropping while I was collecting predators and running these experiments during the fall of 2019. Temperatures in Hattiesburg, MS, averaged 34 °C during the day to 21 °C at night in mid-September, 34 to 20 °C in late September to early

October, 26 to 15 °C in mid-October, and 22 to 10.5 °C in late October to early November (accuweather.com). Photoperiod ranged from 12 hr, 20 min of daylight in mid-September to 10 hr, 55 min in early November of 2019 (timeanddate.com). Insects are ectothermic, therefore lower temperatures likely lower their overall activity as well (Culler et al., 2014; Gresens et al., 1982; Inoda, et al., 2007). For example, Pandian et al. (1979) ran experiments examining mosquito larvae consumption by dragonfly nymphs under different temperatures under laboratory conditions. This study found that at 10°C, one mosquito was eaten on average, however eight mosquitoes were eaten on average at 35°C (with a rise in prey consumption in intermediate temperatures) (Pandian et al., 1979). Calosi et al. (2007) found that diving beetle adults surface more frequently and have shorter dive times in warmer temperatures, which means that they might have more time to forage and hunt prey in cooler temperatures. Inoda et al. (2007) showed that lower temperatures are required for the predaceous diving beetle species *Dytiscus sharpi* to prompt reproduction behavior, which might shift priorities away from foraging. Few studies have examined the effects of time constraint by shorter photoperiod on the predation rates of the aquatic insects used in my experiments. De Block and Stoks (2003) found that temperatures affect foraging behavior of damselfly larvae, with damselflies in 18 ºC eating less than in 22 ºC and 26 ºC. In this same study, they found no significant effects of photoperiod on damselfly foraging activity (De Block and Stoks, 2003). Experiments by Johansson and Rowe (1999) witnessed an increase in foraging activity by damselflies in shorter photoperiods. In contrast, Johansson et al. (2001) did not find any significant changes in damselfly foraging behavior in relation to photoperiod changes. Norling (1984) describes how both temperature and photoperiod are important cues of

northern latitude odonates that diapause over winter, so shorter days and colder temperatures will cause an increase in inactivity in some species. With the above examples of temperature-dependent predation rates in mind, it is possible that perhaps if these experiments were conducted earlier in the summer of 2019, the predators used would have been more voracious. A third possibility is that the predators in my study are not as effective at lowering mosquito numbers in my mesocosms as I had predicted. However, I find this last explanation the weakest, as past research suggests that there should have at least been significant differences in mosquito emergence when comparing negative controls of no predators to every other treatment (Connelly and Carlson, 2009; Kumar and Jiang-Shiou, 2006; Mogi, 2007; Shaalan and Canyon, 2009; Walton, 1996). While predators like odonates and beetles may not eliminate mosquitoes in real-world conditions, (as mosquito larvae are only one of many potential prey items) they definitely regulate the number of mosquitoes produced from a site when present (Connelly and Carlson, 2009; Kumar and Jiang-Shiou, 2006; Mogi, 2007; Shaalan and Canyon, 2009; Walton, 1996).

I also examined the occurrence of IGP among individuals from my predator groups. I hypothesized that IGP would occur more often in the absence of mosquito larvae as prey. Although I only had five replicates of each predator combination, I witnessed IGP between dragonflies and *Laccophilus* larvae and between damselflies and *Laccophilus* larvae but only in the absence of shared prey. This suggests that in my mesocosms, intraguild predation was possible but not likely to occur given that mosquito larvae were available as prey. Predation did not occur between dragonflies and damselflies, damselflies and *Laccophilus* adults, or *Laccophilus* adults and *Laccophilus* larvae. In

control replicates of my behavioral experiment, dragonfly juveniles generally spent most of their time in the bottom 1/3 of the aquarium (90.8%) and usually not on plant structure (65.4% on the floor and 21.7% on plants). Damselfly nymphs were most often resting (96.7%) while perched on plants (73.0% on plants and 25.8% on the floor) and spent 25.0% of the time near the top 1/3 of the water column and 62.5% near the bottom 1/3. *Laccophilus* adults were the most active predator; swimming 25.0% of the time and were often attached to the water's surface siphoning air (51.3%). *Laccophilus* adults were also less often seen at the bottom 1/3 of the water column (18.3%) and were observed walking or perching on plants (57.1%) when not swimming or resting at the surface. *Laccophilus* larvae were on the floor of the aquarium 44.6% of the time, spending the rest of the time on plants (25.8%) or at the surface 28.3), rarely on the wall (1.2%). These differences in resting location preference of predators in control observations are the best explanation for why damselflies and beetle adults were not consumed by dragonflies, given the large size differences. Knowing their behavior and location preferences, damselflies and beetle adults were more likely to interact with each other (especially in these tri-pour cups with smaller widths than the observation aquariums), I expect that no predation occurred due to their similarities in size. It is unlikely that the genera of damselfly used in these experiments (*Ischnura* sp.) would have been able to fit a *Laccophilus* adult in its mouth (since *Laccophilus*' bodies are wider than a late-instar *Ischnura* head), in addition the hard exoskeleton of adult beetles may make them difficult for juvenile odonate to consume. *Laccophilus* adults would have difficulty killing *Ischnura* as well, since they have chewing mouthparts and ability to kill and eat prey depends on their mouth size

(Culler et al., 2014). If IGP were to occur in my mesocosms, I would expect dragonflies to be the predator in most cases given their larger size.

I compared the number of mosquitoes left alive between control treatments of mosquitoes only, and the predators with mosquito larvae treatments. These comparisons were made to ensure mosquito predation was occurring in IGP trials, and their mortality was not due to any other outside factors. There were no significant differences between controls (mosquitoes only) and predators with mosquitoes in all IGP tests. Although I only observed IGP occurring between odonates and beetle larvae, past literature has shown that predation can happen (including cannibalism) among all of my predator groups, and is dependent on factors like body size, age, food scarcity, and predator density (Anholt, 1994; Bofill and Yee, 2019; Culler et al., 2014; Culler and Lamp, 2009; Deding, 1988; Hicks, 1994; Hopper et al., 1996; Johnson and Jakinovich, 1970; Van Buskirk, 1989 and 1992; Wissinger, 1988; Yee, 2010).

There was no predation between *Laccophilus* adults and larvae, which might be due to them being of the same species, having not been starved long enough to induce cannibalism, or having differences in behavior and location preference as well (adults are more mobile, while larvae are sit-and-wait predators when perches are present (Bofill and Yee, 2019). However, my methods are based on Bofill and Yee (2019), and in their case there were two instances of *Laccophilus* adults killing juveniles of the same species. Gut content analyses by Deding (1988) and Hicks (1994) have shown that adult dytiscids will consume larvae of their own species. Although I did not test IGP between predators of the same group, cannibalism does occur among dytiscid larvae (Culler and Lamp, 2009). Yee (2010) found that predaceous diving beetle larvae (Genus: *Rhantus*) are more likely

to eat each other when perching structure is absent, and when body size differences between individuals is greater. Predation among dytiscid adults of the same size is unlikely due to their feeding mode and mouth size (Culler et al., 2014; Johnson and Jakinovich, 1970). Dragonfly larvae can cannibalize each other, but it is more likely to occur between different instars, and in denser populations (Van Buskirk, 1989 and 1992; Wissinger, 1988). Cannibalism among damselfly larvae exists as well, but most likely to occur between different sized instars, at higher densities, and when other prey is absent (Anholt, 1994; Hopper et al., 1996). Anholt (1994) also found that the presence of plant structure reduces the predation rates of larger instar damselflies on smaller instars. The above examples suggest that although IGP and cannibalism might have occurred in my mesocosms it would have been uncommon due to low predator density (one insect per 3 L water), equal size/instar of duplicate taxa, presence of mosquito larvae as food, and presence of live and dead plant structure.

As I had expected in my contamination tests, there were no significant differences in mosquito larval survival among mosquitoes reared from water samples from each mesocosm. This helps support that differences in mosquito emergence were likely due to predation and not influenced by chemical contamination.

For my field mesocosm trials assessing how predators exposed to larvicides, I did not collect the predators at the end of my trials as it would have been unfeasible to sift through all of the plant detritus in a timely manner and simultaneously get an accurate count of all live and dead adults. Thus, I have no accurate information on the long-term survival of these predators after they were introduced. If all my predators had died within a few days, that could be an explanation of why I did not see differences in mosquito

emergence. However, I do not believe this was the case based on my long-term survival experiments from Chapter II, where the only significant differences in mortality were seen in *Laccophilus* adults that died during high and medium concentrations of SF treatments. In addition, cursory examinations of the mesocosms while I was draining them did yield the presence of predators, although again I did not quantify their abundance.

The mesocosms in this experiment were designed to simulate a shallow roadside ditch including the presence of plants. However, the invertebrate community and artificial introduction of nutrients are not accurate to real-world scenarios. Future studies could investigate the effectiveness of mosquito predators as they naturally occur in bodies of water that are commonly treated with larvicides (e.g., ephemeral pools, roadside ditches), or examine colonization of insects in open mesocosms before and after the addition of these chemicals. The more we know about the complex interactions that occur among predaceous aquatic insects, mosquitoes, other invertebrates, and vertebrates that inhabit temporary aquatic environments; the better equipped people will be for reducing medically important mosquito species that emerge from habitats near human populations.

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